


FORM PTO 1390 (REV 5-93)		US DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY DOCKET NUMBER 2001_0531A	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. §371				U.S. APPLICATION NO. 09/830647 (if known, see 37 CFR 1.61) NEW	
International Application No. PCT/JP99/06076		International Filing Date November 1, 1999		Priority Date Claimed October 30, 1998	
Title of Invention HUMAN H37 PROTEIN AND cDNA ENCODING THE PROTEIN					
Applicant(s) For DO/EO/US Kenichi ARAI and Hisao MASAI					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. §371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. §371. 3. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. §371(b) and PCT Articles 22 and 39(1). 4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. §371(c)(2)) a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US) 6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. §371(c)(2)). ATTACHMENT A 7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. §371(c)(3)). a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19. 9. <input checked="" type="checkbox"/> An unexecuted oath or declaration of the inventor(s) (35 U.S.C. §371(c)(4)). ATTACHMENT B 10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371(c)(5)). Items 11. to 14. below concern other document(s) or information included: 11. <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. ATTACHMENT C 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 14. <input type="checkbox"/> Other items or information:					

THE COMMISSIONER IS AUTHORIZED
 TO CHARGE ANY DEFICIENCY IN THE
 FEE FOR THIS PAPER TO DEPOSIT
 ACCOUNT NO. 23-0975.

U.S. APPLICATION NO. 097830647 NEW		INTERNATIONAL APPLICATION NO. PCT/JP99/06076		ATTORNEY'S DOCKET NO. 2001 0531A					
15. [X] The following fees are submitted BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee nor international search fee paid to USPTO and International Search Report not prepared by the EPO or JPO \$1000.00 International Search Report has been prepared by the EPO or JPO \$ 860.00 International preliminary examination fee not paid to USPTO but international search paid to USPTO \$ 710.00 International preliminary examination fee paid to USPTO but claims did not satisfy provisions of PCT Article 33(1)-(4) \$ 690.00 International preliminary examination fee paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$ 100.00 ENTER APPROPRIATE BASIC FEE AMOUNT =				<table border="1" style="width:100%; border-collapse: collapse;"> <tr> <th style="width:50%;">CALCULATIONS</th> <th style="width:50%;">PTO USE ONLY</th> </tr> <tr> <td style="height: 100px; vertical-align: bottom;">\$860.00</td> <td></td> </tr> </table>		CALCULATIONS	PTO USE ONLY	\$860.00	
CALCULATIONS	PTO USE ONLY								
\$860.00									
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$					
Claims	Number Filed	Number Extra	Rate						
Total Claims	26 -20 =	6	X \$18.00	\$108.00					
Independent Claims	4 - 3 =	1	X \$80.00	\$80.00					
Multiple dependent claim(s) (if applicable)			+ \$270.00	\$270.00					
TOTAL OF ABOVE CALCULATIONS =				\$1,318.00					
<input type="checkbox"/> Small Entity Status is hereby asserted. Above fees are reduced by 1/2.				\$					
SUBTOTAL =				\$1,318.00					
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				+	\$				
TOTAL NATIONAL FEE =				\$1,318.00					
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40 per property				+	\$				
TOTAL FEES ENCLOSED =				\$1,318.00					
				Amount to be refunded	\$				
				Amount to be charged	\$				
a. [X] A check in the amount of \$1,318.00 to cover the above fees is enclosed. A duplicate copy of this form is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. 23-0975 in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-0975.									
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.									
19. CORRESPONDENCE ADDRESS <div style="text-align: center;">  000513 PATENT TRADEMARK OFFICE </div>			By: <u>Warren M. Cheek, Jr.</u> Warren M. Cheek, Jr., Registration No. 33,367 WENDEROTH, LIND & PONACK, L.L.P. 2033 "K" Street, N.W., Suite 800 Washington, D.C. 20006-1021 Phone: (202) 721-8200 Fax: (202) 721-8250 April 30, 2001						

[CHECK NO. 44259]

[2001_0531A]

20/PRTS

09/830647
JC18 Rec'd PCT/PTO 30 APR 2001

1

DESCRIPTION

Human H37 protein and cDNA encoding the Protein

5

Technical Field

10

This application relates to human H37 protein and to cDNA encoding the protein. More particularly, this application relates to human H37 protein that is an activity-controlling subunit for the protein Cdc7 controlling the replication of human cells; to human gene encoding the protein; to an antibody to the H37 protein; and to a method for controlling the proliferation of human cells using such genetic engineering material and antibody.

15

Background Art

20

Proliferation of cells is initiated when a liquid factor called a growth factor is bound to the receptor on the cell surface and a signal for proliferation is transmitted into the cell. Accordingly, for an artificial induction of proliferation of incubated cells, methods where an excessive amount of a growth factor is added to a cell medium, where a receptor which is not inherently owned by the said cell is expressed on the cell surface and a factor which is specific for the receptor is added to a medium, etc. have been carried out. Further, in suppressing the cell proliferation, methods where competing molecule, antagonist or the like to the receptor protein is added to the medium to suppress the binding of the receptor to the growth factor, etc. have been carried out.

25

30

On the other hand, in the case of the cell where a proliferation signal is issued by binding of the receptor to the growth factor, a cycle in which its genomic DNA is replicated, uniformly distributed to daughter cells and then divided is repeated. Such a cycle is called "cell cycle" especially for eukaryotes. The cell

09830647.073001

cycle is basically classified into four phases. Thus, S phase when chromosomal DNA is replicated; M phase when the replicated chromosome is divided by a spindle body and then cytoplasm is divided; G1 phase which is a period from M phase finishes until S phase begins; and G2 phase which is a period from S phase finishes until M phase starts. Transition from G1 phase to S phase is particularly strictly controlled and DNA replication takes place only once in S phase.

It has been confirmed from the studies in yeast and higher eukaryotic cells that cyclin-dependent kinase plays a critical role in the cell cycle progression (Nature 292:558-560, 1981; Cell 66:731-742, 1991; Nature 349:338-393, 1991; Science 257:1958-1961, 1992; Bioassays 17:471, 1995). Further, from a genetic analysis in yeast, it has been clarified that another serine/threonine kinase plays an essential role in the initial stage of S phase (G1/S transition). Thus, characterization of cdc7 mutation which was isolated as one of the cell division cycle mutants (J. Mol. Biol. 59:183-194, 1971), it revealed that the Cdc7 protein kinase functions immediately prior to chromosomal replication and that, during S phase, that is necessary for activation of origins (Mol. Cell Biol. 6:1590-1598, 1986; Genes Dev. 15:480-490, 1998; Genes Dev. 15:491-501, 1998). It has been also clarified that the Cdc7 kinase activity is dependent upon the presence of a regulatory subunit, Dbf4 (Genetics 131:21-29, 1992; Mol. Cell. Biol. 13:2899-2908, 1993). Expression of Dbf4 is periodic and is regulated by both at the transcriptional and post-translational levels (Exp. Cell Res. 180:419-428, 1989). The increase in Cdc7 kinase activity at the G1/S boundary is at least accounted for by the elevated expression of Dbf4 in late G1 phase (Mol. Cell. Biol. 13:2899-2908, 1994; Exp. Cell Res. 180:419-428, 1989). In addition, since Dbf4 interacts with replication origins in vivo (Science 265:1243-1246, 1994), it has been suggested that the Cdc7 may trigger S phase by directly activating the replication initiation complex assembled at the origins.

In addition, the inventors of this application had already isolated kinases related to yeast Cdc7 from *Schizosaccharomyces pombe*, *Xenopus*, mouse and human (J. Biol. Chem. 273:23248-23257, 1998; EMBO J. 16:4340-4351, 1997;

EMBO J. 14:3094-3104, 1995), and pointed out that eukaryotic chromosomal replication is regulated by a conserved mechanism involving this family of kinase.

From the findings in yeast and higher eukaryotes as mentioned above, it is expected that an artificial control of the cell proliferation is possible by means of the regulation of the Cdc7 kinase activity in cells, which is entirely other means than conventional methods using operation of growth factor/receptor binding.

However, the inventors had also found that the putative human homolog of Cdc7, huCdc7, possesses only a very low level of kinase activity when overexpressed in mammalian cells while a baculovirus expressed form of huCdc7 is inactive.

Under such circumstances, the inventors of this application have investigated a human cDNA library with a presumption of the presence of a regulatory subunit for human Cdc7, succeeded in isolating the cDNA encoding a novel protein which regulates the kinase activity by binding to huCdc7 and named the said protein encoded in this cDNA as H37 protein.

An object of the invention is to provide the novel protein obtained by the inventors in an industrially applicable form.

Another object of the invention is to provide a human gene encoding the protein and to provide a material for genetic operation including cDNA derived from the gene, an antibody to the protein.

Still another object of the Invention is to provide a method for the artificial control of proliferation of human cells using the above material for genetic operation.

Disclosure of the Invention

This application provides human H37 protein having an amino acid sequence of SEQ ID NO: 1 or NO: 2.

This application also provides human H37 protein having an amino acid sequence where one or more amino acid residue(s) in the amino acid sequence of SEQ ID NO: 1 or NO: 2 is/are deleted therefrom, substituted therefor or added thereto.

This application further provides a human gene encoding the above-mentioned human H37 protein; cDNA of said human gene which has a base sequence of SEQ ID NO: 3 or NO: 4; and DNA fragment comprising a partial sequence of those cDNAs.

This application furthermore provides a recombinant vector having the above-mentioned cDNA and an antibody against the human H37 protein.

This application still further provides a method for promoting the proliferation of cells, which comprises introducing the above-mentioned cDNA or the DNA fragment together with expression regulatory sequence into cell, and a method for suppressing the proliferation of cells, which comprises introducing the above-mentioned antibody into the cell.

Brief Description of Drawings

Fig. 1 is the result of a western blotting measuring the coimmunoprecipitation of H37 with huCdc7 expressed in mammalian cells. Lanes 1-4, immunoprecipitates; lanes 5-7, whole cell extract. Upper and middle panels, immunoprecipitated with anti-huCdc7 antibody No. 1; lower panel, immunoprecipitated with anti-myc antibody. Extracts were prepared from Cos7

cells transfected with huCdc7 in combination with H1 (lanes 2 and 5), H18 (lanes 3 and 6), H37 (lanes 4 and 7) or huCdc7 alone (lane 1). Western blotting was conducted with anti-myc antibody (upper panel) or an anti-huCdc7 antibody No.1 (middle and lower panel).

5

Fig. 2 is a result of the western blotting measuring antibodies against H37 protein and association of huCdc7 and H37 *in vivo*. Nuclear extracts prepared from Cos7 cells transfected with myc-tagged H37 cDNA were blotted with anti-H37C (lane 1), anti-H37N (lane 2), anti-H37Cpep (lane 3) or anti-myc (lane 4) antibody. The arrow indicates the myc-tagged H37 protein, which carries 63 amino acids derived from 5' non-coding region in addition to the myc-tag. Immunoprecipitations from CEM extracts using either anti-huCdc7Cpep (lanes 5 and 6) or anti-H37Cpep (lanes 7 and 8) were separated on gel electrophoresis and blotted with huCdc7 monoclonal antibody (4A8). The symbols - and + indicates absence and presence, respectively, of each antigen peptide during immunoprecipitation. Lanes 9-13; immunoprecipitates, prepared from nuclear extracts of HeLa cells by anti-huCdc7 No.1 (lane 9), anti-huCdc7 monoclonal antibody 4A8 (lane 10), anti-H37C (lane 11), anti-H37N (lane 12) or anti-H37Cpep (lane 13), were blotted with anti-H37Cpep.

20

Fig. 3 shows the result of immunoprecipitation by the use of anti-huCdc7 antibody No.1 (lanes 1-5) or anti-myc antibody (lanes 6-10) from the extract of Cos7 cells transfected with myc-tagged H37 alone (lanes 1 and 6), or together with wild-type huCdc7 (lanes 2 and 7), or kinase negative huCdc7 (lanes 3 and 8). Wild-type huCdc7 alone (lanes 4 and 9) and kinase negative huCdc7 alone (lanes 5 and 10) were included as control.

25

Fig. 4 shows mobility shift of H37 induced by coexpression of wild-type huCdc7. Extracts were prepared from Cos7 cells expressing either wild-type or kinase negative huCdc7 together with myc-tagged H37. Immunoprecipitates with anti-huCdc7 antibody No. 1 (lanes 1 and 2) or anti-myc antibody (lanes 3 and 4) were blotted with anti-myc antibody (upper) or anti-huCdc7 antibody (lower).

30

In all cases, samples were run on 8% SDS-PAGE.

Fig. 5 shows an amino acid sequence of the full-length H37 protein which is as same as the SEQ ID NO: 1.

5

Fig. 6 shows schematic representation of conserved regions between Dbf4 and H37. The double-arrowed region on Dbf4 was reported to be sufficient for interaction with huCdc7. Solid and gray double-arrowed region on H37 indicates the portion essential for interaction with huCdc7 or that sufficient for activation of huCdc7 kinase activity, respectively.

10

Fig. 7 is a schematic representation of N-terminal and C-terminal deletion derivatives of H37 proteins. The number at the end of each bar indicates the portion of the amino acid (corresponding to SEQ ID NO: 1) at the deletion endpoint. The striped region indicates Dbf4 motif-C.

15

Fig. 8 shows lacZ activity of H37 deletion derivatives in two-hybrid assay with huCdc7.

20

Fig. 9 shows the result of antibody coprecipitation method in which H37 deletion derivatives and huCdc7 were co-expressed in COS7 cells and a complex formation was measured.

25

Fig. 10 (a) is the result of northern analysis of H37 mRNA expression in various tissues, and (b) is the result of a northern analysis of H37 mRNA expression in various cancer cell lines.

30

Fig. 11 shows the results where WI38 cells in a resting phase are stimulated by addition of 10% of serum and DNA contents at various stages were analyzed by means of FACS.

Fig. 12 shows the result of northern analysis of H37 and huCdc6

expression by using RNA extracted from cells of Fig. 11 (upper), and the graphs showing the relative expression amount of each mRNA (middle and lower).

Fig. 13 shows graphs showing each cell cycle fraction of human CEM cells
5 fractionated by an elutriation method.

Fig. 14 shows the result of northern analysis for expression of H37 and cyclin E in each fraction of Fig. 13 (upper), and also shows the graphs showing the relative expression amounts of each mRNA (middle and lower).
10

Fig. 15 shows the result of FACS analysis for DNA content in which HeLa cells were stopped at latter G2 phase using nocodazole and then the cell cycle was synchronously shifted.

Fig. 16 shows the result northern analysis for expression of H37 and cyclin E in each cell cycle of Fig. 14 (upper), and also shows the graphs showing the relative expression amounts of each mRNA (middle and lower).
15

Fig. 17 shows H37 localization in cells measured by indirect immunofluorescent technique. The antibodies used are anti-H37C (A), anti-H37N (C) and a control antibody (E) while B, D and F were pictures stained with DAPI.
20

Fig. 18 shows kinetics of induction of DNA replication after serum stimulation of KD cells as measured by BrdU incorporation.
25

Fig. 19 shows the results in which KD cells, synchronized by serum starvation, were microinjected with various antibodies at 12 hours after serum stimulation, and then the rate of the cells incorporating the BrdU after 16 hours was measured. The numbers show the rate of the cells which are conducting the
30 DNA synthesis.

Fig. 20 is microscopic pictures of examples of cells into which a mixture of

anti-H37Cpep antibody and antigen peptide was microinjected. The pictures show the incorporated BrdU (upper), injected antibody (middle) and cells (lower).

5

Best Mode for Carrying Out the Invention

The human H37 protein of this invention has the amino acid sequence of SEQ ID NO: 1 and is a protein molecule encoded in the sequence region from 518th to 2541st of cDNA whose base sequence is shown in SEQ ID NO: 3. The H37 protein of this invention also has the amino acid sequence of SEQ ID NO: 2 and is a protein encoded in the sequence region from 518th to 1222nd of cDNA whose base sequence is shown in SEQ ID NO: 4. SEQ ID NO: 3 and NO: 4 are cDNAs derived from mRNA transcribed from the same genomic gene but the cDNA of SEQ ID NO: 4 is in a different splicing form from SEQ ID NO: 3, and there is a deletion from 1199th to 1259th of SEQ ID NO: 3.

Those H37 proteins can be prepared by known methods such as isolation from human organs and cell lines, chemical synthesis of peptide based upon the amino acid sequence provided by this invention, and a recombinant DNA technique using cDNA fragments provided by this invention. For example, in case the H37 protein is prepared by means of a recombinant DNA technique, RNA is prepared from a vector having the cDNA fragment of this invention by an in vitro transcription and then an in vitro translation is carried out using the above as a template whereby an expression in vitro is possible. In addition, when the translational region is recombined to a suitable vector by a known method, it is possible to express the H37 protein encoded in cDNA in a large quantity in *Escherichia coli*, *Bacillus subtilis*, yeast, insect cells, animal cells, etc.

When the human H37 protein of this invention is expressed in a microorganism such as *E. coli*, the translational region of cDNA of this invention is inserted into an expression vector having, for example, promoter, ribosome binding site, cDNA cloning site, terminator, origin replicable in microorganism, etc.

whereupon a recombinant expression vector is prepared, then host cell is transformed using the said expression vector and the resulting transformant is incubated whereupon H37 protein encoded in cDNA can be produced in a microorganism in a large quantity. Alternatively, it can be expressed as a fusion protein with other protein. The resulting fusion protein is cleaved by an appropriate protease whereupon only the protein portion encoded in cDNA can be obtained.

When the human H37 protein of this invention is expressed in animal cells, the translational region of cDNA of this invention is recombined with expression vector for animal cells having, for example, promoter, poly(A) addition site, and splicing site, and then introduced into the animal cells whereupon the H37 protein of this invention can be expressed in animal cells.

The human H37 protein obtained by the method as mentioned above can be used as an antigen for the preparation of antibody for suppressing the proliferation of cells through inhibition of the kinase activity of huCdc7.

In addition, as will be confirmed by the Examples later, the human H37 protein of this invention has almost no similarity in terms of structure to cyclins which have been elucidated up to now, but it may be regarded as a cyclin-like associating factor for huCdc7 kinase in such respects that its expression is regulated by a cell cycle and that, as a result of binding to an huCdc7 catalytic subunit, its kinase activity can be activated. Accordingly, since the H37 protein is believed to be a very important target factor in a signal transduction pathway for the cell proliferation induced by a growth factor, elucidation of how the expression of the H37 protein or activity thereof is regulated by the signal of cell cycle in G1/S phases is expected to provide a big and novel finding for clarifying the molecular mechanism of cell cycle control of cell replication in animal cells.

The human H37 protein of the invention contains peptide fragments (five or more amino acid residues) containing any partial amino acid sequence of SEQ

ID NO: 1 or NO: 2. Such peptide fragments can be also used as an antigen for the preparation of antibody.

The gene of the invention is a human gene encoding the above-mentioned
5 human H37 protein and can be isolated from known genomic libraries using, for example, the cDNA of this invention or a partial sequence thereof as a probe.

The cDNA of the invention is characterized in having a base sequence of
SEQ ID NO: 3 or NO: 4, and can be cloned, for example, from cDNA libraries
10 derived from human cells. cDNA is synthesized using poly(A)⁺ RNA extracted from human cells as a template. The human cells may be either those excised from human body by a surgical operation or cell lines. The cDNA can be prepared by known methods such as Okayama-Berg method (Okayama, H and Berg, P., Mol. Cell. Biol., 2:161-170, 1982), Gubler-Hoffmann method (Gubler, U.
15 and Hoffman, J. Gene, 25:263-269, 1983) and Capping method (Kato, S. et al., Gene, 150:243-250, 1994).

The human H37 protein of the invention is expressed in any tissue except brain and kidney and, therefore, when the human cDNA library prepared from
20 human cells is screened using an oligonucleotide probe synthesized based on the base sequence of cDNA in SEQ ID NO: 3 or NO: 4, it is possible to easily prepare the same clone of the invention. It is also possible that the desired cDNA is synthesized by a polymerase chain reaction (PCR) using such an oligonucleotide as a primer.

25

In general, polymorphism due to the difference among individuals is frequently noted in human gene. Accordingly, cDNA of SEQ ID NO: 3 or NO: 4 where one or more nucleotide(s) is/are added thereto and/or deleted therefrom and/or other nucleotide(s) is/are substituted therefor is also covered by the
30 invention.

Similarly, the protein where one or more nucleotide(s) is/are added

thereto and/or deleted therefrom and/or other nucleotide(s) is/are substituted therefor as a result of those modifications is also covered by the invention so far as it has an activity of the protein having an amino acid sequence of SEQ ID NO: 1 or NO: 2. Further, variant protein where one or more nucleotide(s) is/are added thereto and/or deleted therefrom and/or other nucleotide(s) is/are substituted by an artificial means is covered by this invention as well.

The DNA fragment of the invention covers a cDNA fragment (10 bp or more) containing any partial base sequence of SEQ ID NO: 3 or NO: 4, or a cDNA fragment comprising an antisense strand thereof.

Antibody against human H37 protein of the invention can be prepared as a polyclonal antibody or a monoclonal antibody by a known method using protein per se or a partial peptide thereof.

A method for promoting the cell proliferation according to the invention is carried out by such a manner that a recombinant DNA comprising the cDNA having a base sequence of SEQ ID NO: 3 or NO: 4, or comprising a partial sequence thereof (such as a DNA fragment encoding 419 amino acid sequence region at the C terminal as shown in Example 3) and an expression-regulatory sequence (promoter and/or enhancer sequence for animal cells) is introduced into animal cells whereby the H37 protein having an amino sequence of SEQ ID NO: 1 or NO: 2 is excessively expressed in cell nuclei. Introduction of the recombinant DNA into cells can be carried out by a known method such as a calcium phosphate method, a method where ribosome and erythrocyte ghost are used, an electroporation method, a method where retrovirus or adenovirus is used as a vector and a microinjection method using a glass pipette. Promotion of cell proliferation as such is useful, for example, in a large-scale preparation of the stem cell that is useful for the therapy of human diseases. Stem cells can be differentiated into other kinds of cells such as blood stem cell and nerve stem cell, and are able to produce a large number of cells of different cell type that constitute the human body. Therefore, transplantation of stem cell in the diseases such as

leukemia is a very important therapeutic means. However, since no liquid factor for self-proliferation of human stem cells without differentiation has been identified yet, the preparation of stem cells in an amount sufficient for the therapy has not been easy. According to the method of this invention, it could be possible that, as a result of manipulating the proliferation program in stem cells, the stem cells can be unlimitedly self-replicated and self-proliferated in vitro. In addition, such a promotion of cell proliferation in vitro could be also useful for the preparation of a large quantity of cells for introduction of gene for genetic therapy by ex vivo means.

The method for the suppression of cell proliferation according to the invention is carried out by injecting the above-mentioned antibody into cells. Alternatively, it may be carried out by inhibiting the expression of the cell-intrinsic H37 protein gene as well. An example thereof is a method where DNA encoding the ribozyme sequence or the antisense sequence to the transcript of the gene is introduced into the cells. Suppression of the cell proliferation as such is expected to provide a novel means for suppressing an excessive proliferation of cancer cells for example.

Examples

The invention will now be further illustrated by way of the following examples although the invention is not limited to those examples.

Example 1.

H37 cDNA labeled with a Flag tag was ligated to the down stream of an SRalpha promoter and introduced into IL-3 (interleukin 3)-dependent proB cell, Ba/F3, to give a stable transformant cell line constitutively expressing the Flag-tagged H37 protein. In this cell line, rate of the cell at the late S phase or G2

phase where DNA synthesis was almost finishing or was finished in the population of the proliferating cells increased as compared with the control parental cell line. On the other hand, the parental cells stopped its proliferation by removal of IL-3 and came into G0 phase but, in the stable transformant cell line, rate of the cells existing in S phase significantly increased even after the removal of IL-3. This result shows that the constitutive production of the H37 protein promotes the shift from G0/G1 phase to S phase, or the progress of S phase itself. It also suggests that the progress of cell cycle can be operated by an artificial expression of the H37 protein or a derivative thereof.

Example 2.

cDNA library of HeLa cell was prepared using a pGAD-GH vector and each vector was introduced into yeast strain CG1945 that has a recombinant plasmid with huCdc7 fused to DNA-binding domain of Gal4. As a result of a screening of about 3×10^5 cells of transformed yeast, five clones where β -galactosidase was positive were obtained. DNA base sequence of the insert was determined and the database was searched whereupon all of them were novel cDNA. Among them, three clones were same having a base sequence of SEQ ID NO: 1. This cDNA was named H37. Other two were single clones and were named H1 and H18, respectively.

The interaction of huCdc7 with protein encoded in those positive clones was further investigated using a production increase system in animal cells. Thus, each expression plasmid of H1, H18 and H37 tagged with myc epitope was transfected to animal cell Cos7 together with a full-length huCdc7 expression plasmid. The result is as shown in Fig. 1. Although the H37 protein was coimmunoprecipitated with an antibody against huCdc7, H1 and H18 proteins were not coprecipitated (cf. lanes 2-4 of the upper panel, Fig. 1). On the contrary, huCdc7 was coimmunoprecipitated by using anti-myc antibody alone in the case of the cells where myc-tagged H37 is co-expressed (cf. lane 4 of the lower lane, Fig.

1). From those results, it has been confirmed that only H37 cDNA encodes a protein efficiently interacting with huCdc7.

Then, in order to investigate the intrinsic H37 protein, each antibody
 5 against N- or C-terminal regions of H37 (anti-H37N antibody or anti-H37C antibody) was prepared. Further, antibody against C-terminal oligopeptide of H37 (anti-H37Cpep antibody) and antibody against C-terminal oligopeptide of huCdc7 (anti-huCdc7Cpep antibody) were also prepared. After that, association of each antibody with intrinsic huCdc7 and H37 in cells was measured. The
 10 result is as shown in Fig. 2. Thus, all of the antibodies to H37 specifically react with the myc-tagged H37 protein of 90 kDa expressed in Cos7 cells (cf. lanes 1-4, Fig. 2). Complex prepared from human CEM cells was able to be coprecipitated using an affinity-purified anti-peptide antibody. It was confirmed by an immunoblotting using anti-huCdc7Cpep antibody that all of huCdc7 and H37
 15 immunoprecipitates contained huCdc7 (lanes 5 and 7, Fig. 2). This interaction of H37 with huCdc7 completely disappeared when antibody and peptide used as antigen for preparing the antibody were subjected to a pre-incubation in advance (cf. lane 8, Fig. 2). In extracts of HeLa cells, both anti-Cdc7 antibody and anti-H37 antibody were able to coprecipitate one polypeptide of 80 kDa that
 20 specifically reacted with anti-H37 antibody (lanes 9-13, Fig. 2).

From the above result, it was found that intrinsic huCdc7 and H37 protein were present in cells as a complex.

Example 3.

In order to investigate whether H37 protein has an ability of activating huCdc7, huCdc7/H37 complex prepared by expression of myc-tagged H37 and
 30 wild-type or kinase negative huCdc7 in Cos cells was immunoprecipitated by anti-huCdc7 antibody or anti-myc antibody and then a kinase reaction in vitro was measured using GST-MCM3 fusion protein as a substrate. The result is as

shown in Fig. 3 and in Fig. 4. In the presence of wild-type huCdc7, phosphorylation in a good efficiency was observed for the MCM3 protein in both immunoprecipitate of anti-huCdc7 antibody and immunoprecipitate of anti-myc antibody (cf. lanes 2 and 7, Fig. 3). Further, two more phosphorylated proteins were observed and they were identified as transfected huCdc7 and myc-tagged H37 (data not shown). Since the phosphorylation as such was not detected at all in kinase negative huCdc7, it was confirmed that kinase activity of huCdc7 acted in the phosphorylation. However, kinase negative huCdc7 was also able to form a complex with H37 protein (cf. lanes 3 and 8, Fig. 3; lanes 2 and 4, Fig. 4). In addition, the mobility of H37 protein on gel electrophoresis was slow when a wild-type huCdc7 was co-expressed and detected as plural bands while such a shift was not observed in kinase negative huCdc7 (cf. lanes 1 and 3, Fig. 4). The band where the mobility was slow disappeared by a treatment with phosphatase and, therefore, it was confirmed to be a perphosphorylated H37 protein (data not shown). In addition, when huCdc7 and H37 protein were co-expressed in insect cells, it was possible to reconstitute a very strong kinase activity which was able to efficiently phosphorylate MCM2 and MCM3 proteins (data now shown).

The above result shows that H37 protein activates huCdc7 kinase and further that H37 protein itself is phosphorylated by huCdc7.

Further, under those experimental conditions, intrinsic H37 protein level was too low and in the case where only huCdc7 catalytic subunit was expressed and, therefore, the kinase activity was little (cf. lanes 4 and 9, Fig. 3). From those facts, it was confirmed that H37 protein encoded regulatory cell unit of huCdc7 and specifically activated its kinase activity.

Example 4.

An amino acid sequence of the H37 protein (SEQ ID NO: 1) was analyzed. The result was that, as shown in Fig. 5 and Fig. 6, an amino acid sequence region

having a homology of 33% with budding yeast Dbf4 was found. This conserved domain (H37 motif C) was also present in the gene similar to H37 identified in mouse, fruit fly and fission yeast (cf. Fig. 6). Further, another amino acid sequence of H37 (H37 motif N) was conserved in the H37-related gene in mouse,
 5 fruit fly and fission yeast. However, the H37 motif N was conserved in a somewhat modified form on the Dbf4 protein of a budding yeast (cf. Fig. 6; data partially not shown).

In order to determine the region on H37 protein essential for binding with
 10 huCdc7, a series of deletion derivatives of H37 at N- and C-terminals as shown in Fig. 7 was prepared, each of them was expressed in yeast as a fusion protein with Gal4 activated domain and an interaction of each deletion derivative with huCdc7 was investigated by means of a two-hybrid assay. The result is as shown in Fig. 8. As a result of deletion at N-terminal, even a deletion of 255 amino acids at
 15 N-terminal did not affect the interaction with huCdc7 (Δ N2). However, when 50 amino acids at N-terminal were further deleted whereupon the H37 motif C was deleted, an interaction with huCdc7 was completely lost (Δ N3).

On the other hand, with regard to deletion from C-terminal, when only 20
 20 amino acids were deleted, the binding ability with huCdc7 decreased to an extent of about 60% (Δ C). In addition, when 243 or 369 amino acids were deleted from the C-terminal (Δ P2 and Δ B), the interaction lowered to an extent of about 10% of the full-length clone. Δ P1 containing only 235 amino acids of N-terminal did not interact with huCdc7. However, 50 amino acids that were commonly present in
 25 Δ B and Δ N2 were not sufficient for an efficient interaction with huCdc7 (data not shown).

A part of the above-mentioned H37 deletion derivatives was co-expressed in COS7 cells together with huCdc7 and it was confirmed by immunoprecipitation
 30 method whether it formed a complex with huCdc7 protein. The result was that, like in the result of a two-hybrid assay, it was confirmed that only H37 deletion derivatives of deltaB and deltaN2 formed a complex with huCdc7 (cf. Fig. 9).

The above result shows that H37 motif-C is essential for an interaction of H37 protein with huCdc7 catalytic subunit but shows that H37 motif C alone is insufficient. In the budding yeast, it was reported already that the region containing H37 motif C was sufficient for binding with Cdc7 (Mol. Cell. Biol. 15:6775-6782, 1996). When an in vitro kinase reaction was carried out using those deletion derivatives, it was found that, although the extent was weak, only 419 amino acids at the C-terminal containing Dbf4 motif-C was sufficient for activation of phosphorylating ability of huCdc7 (data not shown).

Example 5.

Expression patterns of H37 mRNA in various human tissues and cancer cells were investigated by northern blotting. The result is as shown in Fig. 10 (a) and (b). By an H37 cDNA-specific probe, a transcribed product of 2.5 kb was detected in all tissues except brain and kidney and also in all cancer cells. That was contrary to the fact that, in brain and kidney, huCdc7 mRNA was relatively highly expressed (EMBO J. 16:4340-4351, 1997). Among the tissues tested, expression of H37 mRNA was highest in testicles and then in thymus, and it was reported by the inventors already that both of them were the tissues where the expression of huCdc7 catalytic subunit was particularly high as well (EMBO J. 16:4340-4351, 1997). In the testicles, two different RMA bands of 6 kb and 4 kb were also detected (cf. Fig. 10 (a)) although it was ambiguous what the real substances therefor were. It was also confirmed that H37 mRNA was expressed in a very high level in nearly all cancer cells except lung cancer cell A549 (cf. Fig. 10 (b)). That shows an important role of H37 protein in the cells having an active proliferating ability.

Example 6.

In order to investigate whether expression of H37 was regulated by cell cycle, human normal fibroblast cell WI38 was synchronized with G0 phase by means of serum starvation (cf. Fig. 11), total RNA was prepared at various periods after addition of serum and the level of H37 mRNA was investigated by northern blotting. The result is as shown in Fig. 12. H37 mRNA level was low in the cell at the resting stage and, as the cells came near the boundary of G1 and S, it gradually increased and, after 20 hours from the serum addition, it became maximum. The expression pattern shown in Fig. 12 was similar to that of transcribed product of huCdc6 that is known to be induced by growth stimulation (Mol. Cell. Biol. 15:4215-4224, 1995; Proc. Natl. Acad. Sci. USA 94:142-147, 1997).

Further, in order to investigate the changes in expression of H37 in cell cycle, human CEM cells were fractionated by means of elutriation method (cf. Fig. 13) and then a northern blotting was carried out (cf. Fig. 14). It was shown by the result thereof that H37 mRNA was low at G1 phase, increased from the late G1 phase to S phase, highly maintained during S phase and somewhat decreased in G2 phase although it was still kept high. In an experiment where HeLa cells were stopped at G2 phase by nocodazole and the cell cycle was synchronously shifted (cf. Fig. 15), it was also found that H37 mRNA decreased together with shift from G2 to G1 and increased again at shift to S phase (cf. Fig. 16). Similarly, expression of Cdc6 rose together with shift from G1 phase to S phase but there was a difference from H37 in that, as S phase proceeded, it decreased and, at G2 phase it was suppressed to low. The result shows that the expression of H37 mRNA changes even in the proliferating cell cycle, becomes maximum at S phase where it functions and is kept high throughout S phase.

After that, in order to investigate the changes in H37 protein and huCdc7 kinase activity depending upon H37 in cell cycle, amount of H37 protein in K562 cells synchronized with nocodazole was analyzed by western analysis while Cdc7 kinase activity was analyzed by phosphorylation reaction of GST-huMCM2 protein using anti-huCdc7 immunoprecipitate in the same manner as in Fig. 15.

Although H37 protein was not detected from G1/M boundary to G1 phase, it started to be detected at the G1/S boundary and detected throughout S phase. This was in contrast to the fact that cyclin E protein is detected already in G1 phase and, as S phase proceeds, the amount suddenly decreases. On the other hand, huCdc kinase activity was also detected in S phase only corresponding to the level of H37 protein. Further, H37 protein was detected as plural phosphorylated bands in S phase and was thought to be phosphorylated by huCdc7 that was probably associated. On the contrary, level of huCdc7 protein was almost constant throughout cell cycle. This result proves that the level of huCdc7 protein varies in cell cycle and is kept high in S phase whereby huCdc7 kinase activity is also kept high in S phase.

In order to further investigate the expression of H37, intracellular localization of H37 protein in animal cells was measured. The result of indirect fluorescent antibody method using two kinds of H37-specific antibodies was that, in both HeLa and WI38 cells, intrinsic H37 protein was observed as very clear various spots in the nuclei (cf. Fig. 17).

When those results and the intranuclear localization of huCdc7 catalytic subunit already reported by the inventors (EMBO J. 16:4340-4351, 1997) were taken into consideration, it was confirmed that huCdc7/H37 complex was a kinase localized in the nuclei and that its regulatory subunit H37 was expressed depending upon the stage of cell cycle.

Example 7.

Function of the intrinsic H37 protein in the shift from G1 to S in cell cycle was investigated by antibody microinjection method. Antibody against N-terminal 305 amino acids of H37 protein (anti-H37N antibody) and antibody against C-terminal oligopeptide (anti-H37Cpep antibody) were treated by affinity purification and those antibodies were microinjected into normal fibroblast cells

(KD cells) derived from human lip. KD cells were previously arrested at G0 phase by means of serum starvation and then synchronously proceeded in cell cycle by re-addition of serum. Numbers of the BrdU-positive cells, which BrdU is a nucleotide derivative and is incorporated into the cells, were measured whereby it was investigated how much fractions of cells at S phase were present after various stages after addition of serum. The result is as shown in Fig. 18. It was confirmed that the cells started DNA synthesis after about 18 hours from serum addition and that, after 24 hours, almost 90% of the cells were in S phase.

Accordingly, in this Example, antibody was microinjected at the period of after 12 hours after the addition of serum where the cells were still in G1 phase and, at 26 hours when cells would be of in S phase, the cells were fixed and the BrdU-positive cells were measured. The result is as shown in Fig. 19. Although 70% of the cells into which anti-H37N antibody was microinjected did not shift into S phase, there was nearly no effect by the control antibody. Even by the microinjection of the anti-H37Cpep antibody, an effect of inhibiting the shift to S phase which was same as or even more than the anti-H37N antibody was observed. In addition, when anti-H37Cpep antibody and a peptide which was an antigen for the preparation of anti-H37Cpep antibody were simultaneously microinjected into the cells, 70% or more of the cells were shifted to S phase.

Fig. 20 is a staining example of BrdU and microinjected antibody. The antibody was microinjected at the stage when the cells were from the mid to late S phase and, during that period, expression of H37 protein is believed to be low. The microinjected antibody efficiently bound to the freshly synthesized H37 protein and, as a result, it is speculated to inhibit the transfer of H37 protein into nucleus. Those results strongly suggest the function of H37, i.e. the function of huCdc7/H37 complex is required for the progression of S phase of the animal cells.

Industrial Applicability

As fully illustrated hereinabove, the present invention provided human H37 protein, a regulatory subunit for Cdc7 that regulate replication of human cells, human gene encoding this protein as well as cDNA thereof, an antibody against H37 protein, and a method for controlling the proliferation of human cells using those genetic engineering materials and antibody. As a result, it provides potentially novel means for preparation of the necessary amount of stem cells or the like to be used for the therapy of various human diseases or for the suppression of proliferation of cancer cells.

0930647.073004

CLAIMS

1. Human H37 protein having an amino acid sequence of SEQ ID NO: 1.
2. Human H37 protein having an amino acid sequence where one or more amino acid residue(s) in the amino acid sequence of SEQ ID NO: 1 is/are deleted therefrom, substituted therefor or added thereto.
3. Human H37 protein having an amino acid sequence of SEQ ID NO: 2.
4. Human H37 protein having an amino acid sequence where one or more amino acid residue(s) in the amino acid sequence of SEQ ID NO: 2 is/are deleted therefrom, substituted therefor or added thereto.
5. A human gene encoding the human H37 protein of claim 1 or 2.
6. A cDNA of the human gene of claim 5, which has a base sequence of SEQ ID NO: 3 or a base sequence where one or more base(s) in the base sequence of SEQ ID NO: 3 is/are deleted therefrom, substituted therefor or added thereto.
7. A cDNA of the human gene of claim 5, which has a base sequence of SEQ ID NO: 4 or a base sequence where one or more base(s) in the base sequence of SEQ ID NO: 4 is/are deleted therefrom, substituted therefor or added thereto.
8. A DNA fragment comprising a partial sequence of cDNA of claim 6.
9. A DNA fragment comprising a partial sequence of cDNA of claim 7.
10. A recombinant vector having the cDNA of claim 6.
11. A recombinant vector having the cDNA of claim 7.

12. An antibody against the human H37 protein of claim 1 or 2.

13. An antibody against the human H37 protein of claim 3 or 4.

5 14. A method for promoting the proliferation of cells, which comprises introducing the cDNA of claim 6 or the DNA fragment of claim 8 together with expression-regulatory sequences into the cells.

10 15. A method for promoting the proliferation of cells, which comprises introducing the cDNA of claim 7 or the DNA fragment of claim 9 together with an expression regulatory sequence into the cells.

16. A method for suppressing the proliferation of cells, which comprises introducing the antibody of claim 10 into the cells.

15 17. A method for suppressing the proliferation of cells, which comprises introducing the antibody of claim 11 into the cells.

ABSTRACT

This application provides human H37 protein having an amino acid sequence of SEQ ID NO: 1 or NO: 2; human gene encoding the protein; cDNA of
5 the human gene which has a base sequence of SEQ ID NO: 3 or NO: 4; DNA fragment comprising a partial sequence of the cDNA; recombinant vector having the cDNA; antibody against human H37 protein; and a method for controlling the proliferation of cells by introducing the above DNA or antibody into the cells.

T00E70"2490E860

<120> Juman H37 protein and cDNA encoding the protein

<141> 1999-11-01

<150> JP No. 10-311408

<151> 1998-10-30

<160> 4

10 <210> 1

<211> 674

<212> PRT

〈213〉 Homo sapiens

<400> 1

15 Met Asn Ser Gly Ala Met Arg Ile His Ser Lys Gly His Phe Gln Gly

1 5 10 15

Gly Ile Gln Val Lys Asn Glu Lys Asn Arg Pro Ser Leu Lys Ser Leu

20 25 30

Lys Thr Asp Asn Arg Pro Glu Lys Ser Lys Cys Lys Pro Leu Trp Gly

20 35 40 45

Lys Val Phe Tyr Leu Asp Leu Pro Ser Val Thr Ile Ser Glu Lys Leu

50 55 60

Gln Lys Asp Ile Lys Asp Leu Gly Gly Arg Val Glu Glu Phe Leu Ser

65 70 75 80

25 Lys Asp Ile Ser Tyr Leu Ile Ser Asn Lys Lys Glu Ala Lys Phe Ala

85 90 95

Gln Thr Leu Gly Arg Ile Ser Pro Val Pro Ser Pro Glu Ser Ala Tyr

100 105 110

115 120 125

130

145 150 155 160

165

10 180 185 190

195 200 205

210 215 220

225 230 235 240

245 250 255

20 260 265 270

275 280 285

290 295 300

305 310 315 320

325

Asp Phe Val Glu Tyr Glu Lys Asp Thr Pro Lys Lys Lys Arg Ile Lys

240

245

350

Tyr Ser Val Gly Ser Leu Ser Pro Val Ser Ala Ser Val Leu Lys Lys

355

360

365

5 Thr Glu Gln Lys Glu Lys Val Glu Leu Gln His Ile Ser Gln Lys Asp

370

375

380

Cys Gln Glu Asp Asp Thr Thr Val Lys Glu Gln Asn Phe Leu Tyr Lys

385

390

395

400

Glu Thr Gln Glu Thr Glu Lys Lys Leu Leu Phe Ile Ser Glu Pro Ile

10

405

410

415

Pro His Pro Ser Asn Glu Leu Arg Gly Leu Asn Glu Lys Met Ser Asn

420

425

430

Lys Cys Ser Met Leu Ser Thr Ala Glu Asp Asp Ile Arg Gln Asn Phe

435

440

445

15 Thr Gln Leu Pro Leu His Lys Asn Lys Gln Glu Cys Ile Leu Asp Ile

450

455

460

Ser Glu His Thr Leu Ser Glu Asn Asp Leu Glu Glu Leu Arg Val Asp

465

470

475

480

His Tyr Lys Cys Asn Ile Gln Ala Ser Val His Val Ser Asp Phe Ser

20

485

490

495

Thr Asp Asn Ser Gly Ser Gln Pro Lys Gln Lys Ser Asp Thr Val Leu

500

505

510

Phe Pro Ala Lys Asp Leu Lys Glu Lys Asp Leu His Ser Ile Phe Thr

515

520

525

25 His Asp Ser Gly Leu Ile Thr Ile Asn Ser Ser Gln Glu His Leu Thr

530

535

540

Val Gln Ala Lys Ala Pro Phe His Thr Pro Pro Glu Glu Pro Asn Glu

545

550

555

560

FOOZ-0-44900000

Cys Asp Phe Lys Asn Met Asp Ser Leu Pro Ser Gly Lys Ile His Arg

565

570

575

Lys Val Lys Ile Ile Leu Gly Arg Asn Arg Lys Glu Asn Leu Glu Pro

580

585

590

5 Asn Ala Glu Phe Asp Lys Arg Thr Glu Phe Ile Thr Gln Glu Glu Asn

595

600

605

Arg Ile Cys Ser Ser Pro Val Gln Ser Leu Leu Asp Leu Phe Gln Thr

610

615

620

Ser Glu Glu Lys Ser Glu Phe Leu Gly Phe Thr Ser Tyr Thr Glu Lys

10

625

630

635

640

Ser Gly Ile Cys Asn Val Leu Asp Ile Trp Glu Glu Glu Asn Ser Asp

645

650

655

Asn Leu Leu Thr Ala Phe Phe Ser Ser Pro Ser Thr Ser Thr Phe Thr

660

665

670

15 Gly Phe

674

<210> 2

<211> 234

<212> PRT

20 <213> Homo sapiens

<400> 2

Met Asn Ser Gly Ala Met Arg Ile His Ser Lys Gly His Phe Gln Gly

1

5

10

15

Gly Ile Gln Val Lys Asn Glu Lys Asn Arg Pro Ser Leu Lys Ser Leu

25

20

25

30

Lys Thr Asp Asn Arg Pro Glu Lys Ser Lys Cys Lys Pro Leu Trp Gly

35

40

45

Lys Val Phe Tyr Leu Asp Leu Pro Ser Val Thr Ile Ser Glu Lys Leu

T00020-2490E360

5

25

<212> DNA

<400> 3

AATTCGGCAC GAGCTCTCTG AGGCTGCGCC AAGACCTGAA GCGGCGGACC GAGAGCCCGG 60
 GTCTGAGACT GAGAGAGCAA CGGAATGGAG GCGGGGTAGA GGCGGAAACA CAACCTGCAG 120
 GGCCAGAGCG AGGCGCGAGA AGGACGGCGG CGTGAGGGGG CGGGGCGCGC AGCGCGAGAA 180
 GGCAGGCACG AGGGGCGAGC GCGAGGCGGG GCACGGCGCG TGGCGTGAGA CGGGGCGGGG 240
 5 CGCGCGTATC GGCGCCGCGG CCGCGTGACG CGTTTTCAAA TCTTCAACCG CCGCAGCCCA 300
 CTCGTTTGTG CTTTGCGCCT TCCTCCTCCG CGCCTTGGAG CCGGATCCGG CCCCAGAAAC 360
 CCGACCTGCA GACGCGGTAC CTCTACTGCG TAGAGGCCGT AGCTGGCGGA AGGAGAGAGG 420
 CGGCCGTCTT GTCAACAGGC CGGGGGAAGC CGTGCTTTTC CGGCTGCCCG GTGCGACACT 480
 TTCTCCGGAC CCAGCATGTA GGTGCCGGGC GACTGCCATG AACTCCGGAG CCATGAGGAT 540
 10 CCACAGTAAA GGACATTTCC AGGGTGGAAT CCAAGTCAA AATGAAAAA ACAGACCATC 600
 TCTGAAATCT CTGAAAACG ATAACAGGCC AGAAAAATCC AAATGTAAGC CACTTTGGGG 660
 AAAAGTATTT TACCTTGACT TACCTTCTGT CACCATATCT GAAAACTTC AAAAGGACAT 720
 TAAGGATCTG GGAGGGCGAG TTGAAGAATT TCTCAGCAA GATATCAGTT ATCTTATTTT 780
 AAATAAGAAG GAAGCTAAAT TTGCACAAAC CTTGGGTCGA ATTTCTCCTG TACCAAGTCC 840
 15 AGAATCTGCA TATACTGCAG AAACCACTTC ACCTCATCCC AGCCATGATG GAAGTTCATT 900
 TAAGTACCA GACACAGTGT GTTTAAGCAG AGGAAAATTA TTAGTTGAAA AAGCTATCAA 960
 GGACCATGAT TTTATTCCTT CAAATAGTAT ATTATCAAAT GCCTTGTCAT GGGGAGTAAA 1020
 AATTCTTCAT ATTGATGACA TTAGATACTA CATTGAACAA AAGAAAAAAG AGTTGTATTT 1080
 ACTCAAGAAA TCAAGTACTT CAGTAAGAGA TGGGGGCAA AGAGTTGGTA GTGGTGCACA 1140
 20 AAAACAAGA ACAGGAAGAC TCAAAAAGCC TTTTGTAAG GTGGAAGATA TGAGCCAACT 1200
 TTATAGGCCA TTTTATCTTC AGCTGACCAA TATGCCTTTT ATAAATTATT CTATTCAGAA 1260
 GCCCTGCAGT CCATTTGATG TAGACAAGCC ATCTAGTATG CAAAAGCAA CTCAGGTAA 1320
 ACTAAGAATC CAAACAGATG GCGATAAGTA TGGTGAACC TCAATTCAAC TCCAGTTGAA 1380
 AGAGAAGAAG AAAAAAGGAT ATTGTGAATG TTGCTTGCAG AAATATGAAG ATCTAGAAAC 1440
 25 TCACCTTCTA AGTGAGCAAC ACAGAACTT TGCACAGAGT AACCAGTATC AAGTTGTTGA 1500
 TGATATTGTA TCTAAGTTAG TTTTGTACTT TGTGGAATAT GAAAAGGACA CACCTAAAAA 1560
 GAAAAGAATA AAATACAGTG TTGGATCCCT TTCTCCTGTT TCTGCAAGTG TCCTGAAAAA 1620
 GACTGAACAA AAGGAAAAAG TGGAATTGCA ACATATTTCT CAGAAAGATT GCCAGGAAGA 1680

T00E20" 2420E850

TGATACAACA GTGAAGGAGC AGAATTTCTT GTATAAAGAG ACCCAGGAAA CTGAAAAAAA 1740
 GCTCCTGTTT ATTTAGAGC CCATCCCCCA CCCTTCAAAT GAATTGAGAG GGCTTAATGA 1800
 GAAAATGAGT AATAAATGTT CCATGTTAAG TACAGCTGAA GATGACATAA GACAGAATTT 1860
 TACACAGCTA CCTCTACATA AAAACAAACA GGAATGCATT CTTGACATTT CCGAACACAC 1920
 5 ATTAAGTGAA AATGACTTAG AAGAACTAAG GGTAGATCAC TATAAATGTA ACATACAGGC 1980
 ATCTGTACAT GTTTCTGATT TCAGTACAGA TAATAGTGA TCTCAACCAA AACAGAAGTC 2040
 AGATACTGTG CTTTTTCCAG CAAAGGATCT CAAGGAAAAG GACCTTCATT CAATATTTAC 2100
 TCATGATTCT GGTCTGATAA CAATAACAG TTCACAAGAG CACCTAACTG TTCAGGCAAA 2160
 GGCTCCATTC CATACTCCTC CTGAGGAACC CAATGAATGT GACTTCAAGA ATATGGATAG 2220
 10 TTTACCTTCT GGTAATAAC ATCGAAAAGT GAAAATAATA TTAGGACGAA ATAGAAAAGA 2280
 AAATCTGGAA CCAAATGCTG AATTTGATAA AAGAACTGAA TTTATTACAC AAGAAGAAAA 2340
 CAGAATTTGT AGTTCACCGG TACAGTCTTT ACTAGACTTG TTTTCACTA GTGAAGAGAA 2400
 ATCAGAATTT TTGGGTTTCA CAAGCTACAC AGAAAAGAGT GGTATATGCA ATGTTTTAGA 2460
 TATTTGGGAA GAGGAAAATT CAGATAATCT GTTAACAGCG TTTTCTCGT CCCCTTCAAC 2520
 15 TTCTACATTT ACTGGCTTTT AGAATTTAAA AAATGCATAC TTTTCAAGAG TGATAAGGAT 2580
 CATATTCTTG AAATTTTAT AAATATGTAT GGAAATTCTT AGGATTTTTT TACCAGCTTT 2640
 GTTTACAGAC CCAAATGTAA ATATTAATAA TAAATATTTG CAATTTTCTA CAGAATTGAA 2700
 TACCTGTAA AGAAAAATTA CAGAATAAAC TTGTGACTGG TCTTGTTTTA CATTAAAAAA 2760
 AAAAAAAAAA AAAACTCGAG 2780

- 20 <210> 4
 <211> 2719
 <212> DNA
 <213> Homo sapiens
 <400> 4

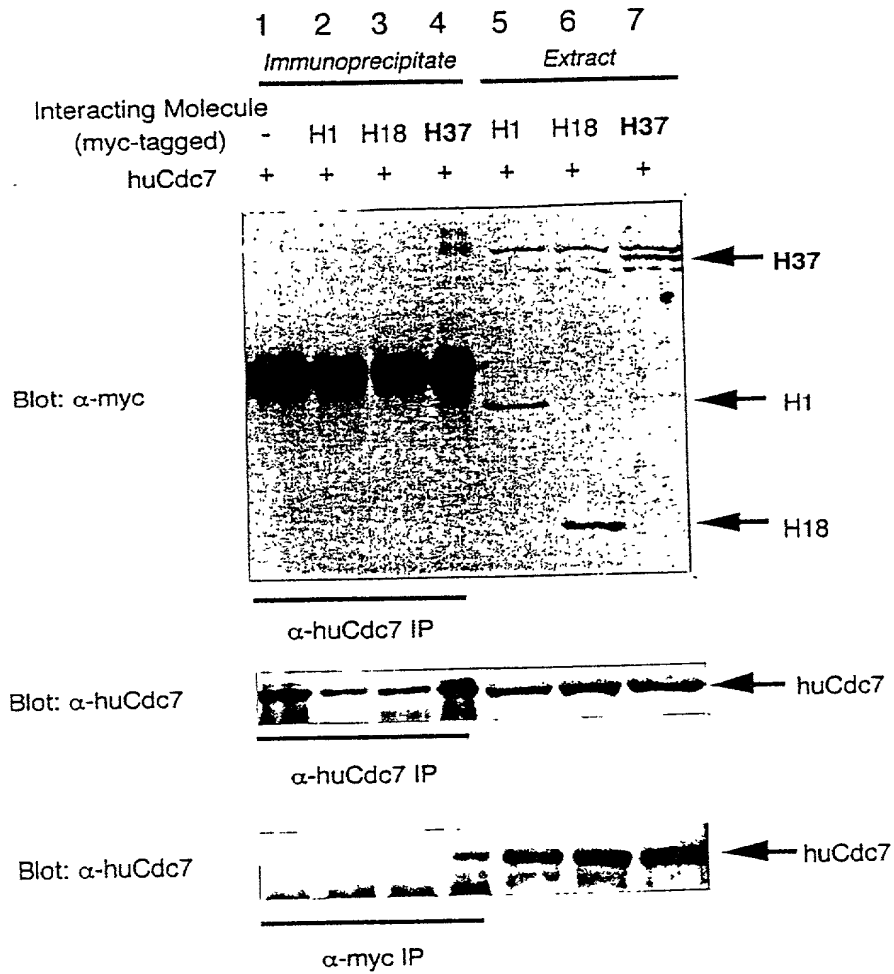
25 AATTCGGCAC GAGCTCTCTG AGGCTGCGCC AAGACCTGAA GCGGCGGACC GAGAGCCCGG 60
 GTCTGAGACT GAGAGAGCAA CGGAATGGAG GCGGGGTAGA GCGGGAACA CAACCTGCAG 120
 GGCCAGAGCG AGGCGCGAGA AGGACGGCGG CGTGAGGGGG CGGGCGCGC AGCGCGAGAA 180
 GGCAGGCACG AGGGGCGAGC GCGAGGCGGG GCACGGCGCG TGGCGTGAGA CGGGCGGGG 240

CGCGCGTATC GCGCGCCGCGG CCGCGTGACG CGTTTTCAAA TCTTCAACCG CCGCAGCCCA 300
CTCGTTTGTG CTTTGCGCCT TCCTCCTCCG CGCCTTGGAG CCGGATCCGG CCCCAGAAAC 360
CCGACCTGCA GACGCGGTAC CTCTACTGCG TAGAGGCCCGT AGCTGGCGGA AGGAGAGAGG 420
CGGCCGTCTT GTCAACAGGC CGGGGGAAGC CGTGCTTTTCG CGGCTGCCCG GTGCGACACT 480
5 TTCTCCGGAC CCAGCATGTA GGTGCCGGGC GACTGCCATG AACTCCGGAG CCATGAGGAT 540
CCACAGTAAA GGACATTTCC AGGGTGAAT CCAAGTCAA AATGAAAAA ACAGACCATC 600
TCTGAAATCT CTGAAAACG ATAACAGGCC AGAAAAATCC AAATGTAAGC CACTTTGGGG 660
AAAAGTATTT TACCTTGACT TACCTTCTGT CACCATATCT GAAAACTTC AAAAGGACAT 720
TAAGGATCTG GGAGGGCGAG TTGAAGAATT TCTCAGCAA GATATCAGTT ATCTTATTTT 780
10 AAATAAGAAG GAAGCTAAAT TTGCACAAAC CTTGGGTCGA ATTTCTCCTG TACCAAGTCC 840
AGAATCTGCA TATACTGCAG AAACCACTTC ACCTCATCCC AGCCATGATG GAAGTTCATT 900
TAAGTCACCA GACACAGTGT GTTTAAGCAG AGGAAAATTA TTAGTTGAAA AAGCTATCAA 960
GGACCATGAT TTTATTCCTT CAAATAGTAT ATTATCAAAT GCCTTGTCAT GGGGAGTAAA 1020
AATTCTTCAT ATTGATGACA TTAGATACTA CATTGAACAA AAGAAAAAAG AGTTGTATTT 1080
15 ACTCAAGAAA TCAAGTACTT CAGTAAGAGA TGGGGGCAA AGAGTTGGTA GTGGTGACA 1140
AAAAACAAGA ACAGGAAGAC TCAAAAAGCC TTTTGTAAG GTGGAAGATA TGAGCCAAAG 1200
CCCTGCAGTC CATTGATGT AGACAAGCCA TCTAGTATGC AAAAGCAAAC TCAGGTTAAA 1260
CTAAGAATCC AACAGATGG CGATAAGTAT GGTGGAACCT CAATTCAACT CCAGTTGAAA 1320
GAGAAGAAGA AAAAAGGATA TTGTGAATGT TGCTTGACA AATATGAAGA TCTAGAACT 1380
20 CACCTTCTAA GTGAGCAACA CAGAACTTT GCACAGAGTA ACCAGTATCA AGTTGTTGAT 1440
GATATTGTAT CTAAGTTAGT TTTTGACTTT GTGGAATATG AAAAGGACAC ACCTAAAAAG 1500
AAAAGAATAA AATACAGTGT TGGATCCCTT TCTCCTGTTT CTGCAAGTGT CCTGAAAAAG 1560
ACTGAACAAA AGGAAAAAGT GGAATTGCAA CATATTTCTC AGAAAGATTG CCAGGAAGAT 1620
GATACAACAG TGAAGGAGCA GAATTCCTG TATAAGAGA CCCAGGAAAC TGAAAAAAG 1680
25 CTCCTGTTTA TTTCAGAGCC CATCCCCAC CCTTCAAATG AATTGAGAGG GCTTAATGAG 1740
AAAATGAGTA ATAAATGTTC CATGTTAAGT ACAGCTGAAG ATGACATAAG ACAGAATTTT 1800
ACACAGCTAC CTCTACATAA AAACAAACAG GAATGCATTC TTGACATTTT CGAACACACA 1860
TTAAGTGAAG ATGACTTAGA AGAACTAAGG GTAGATCACT ATAAATGTAA CATACAGGCA 1920

15

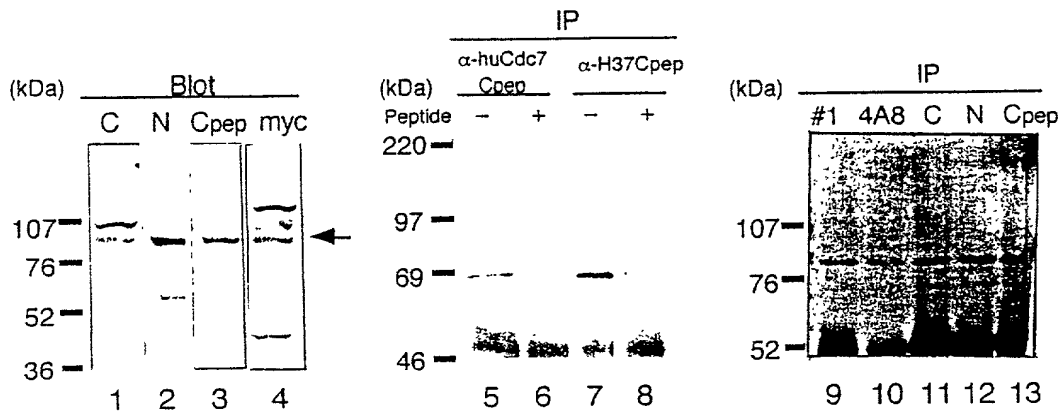
1/20

Fig.1



2/20

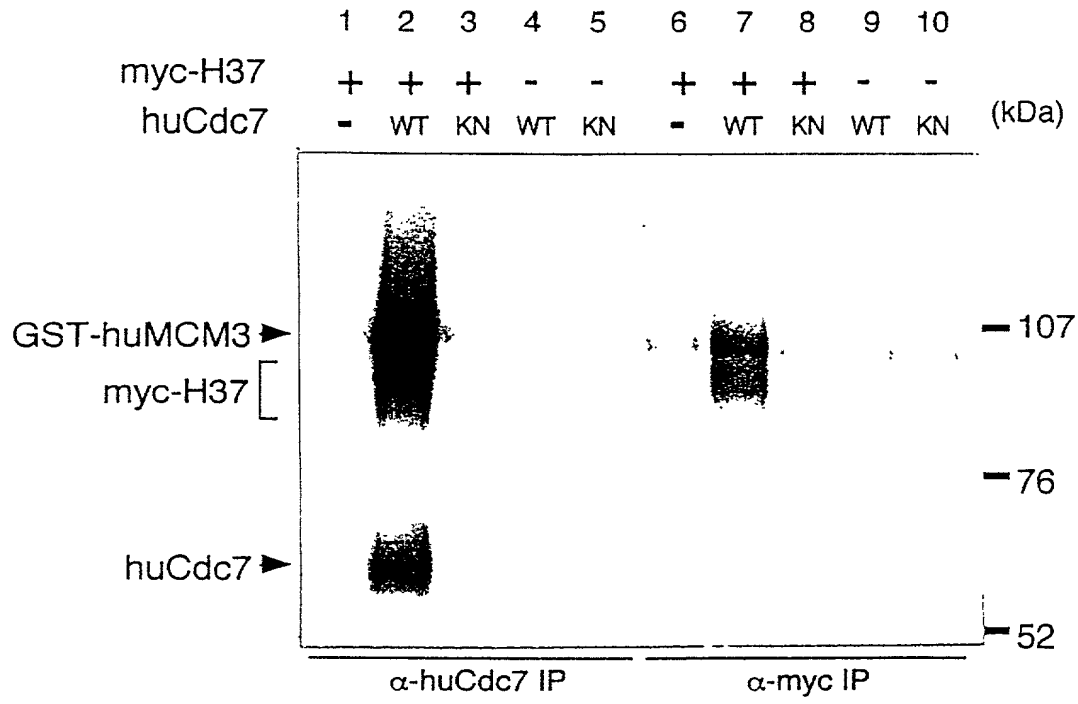
Fig.2



3/20

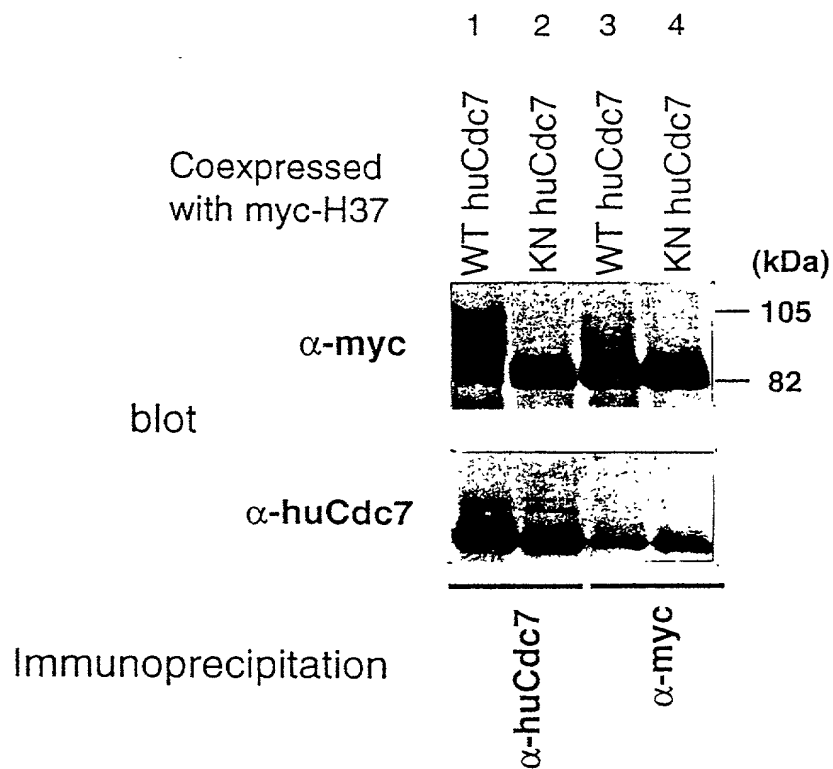
09/830647

Fig.3



4/20

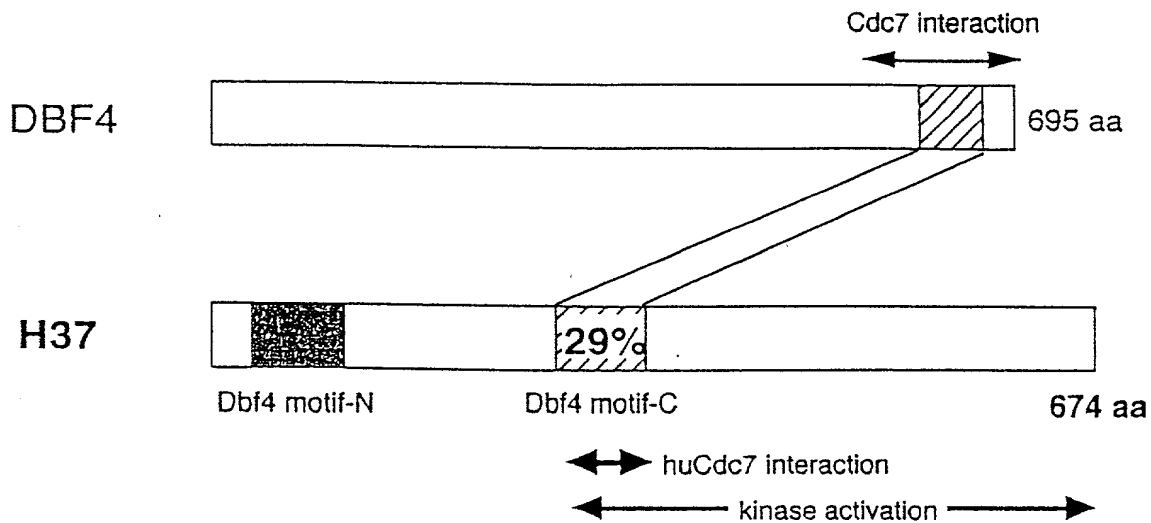
Fig.4



1 10 20 30 40 50 60
MNSGAMRIHSKGFQGGIQVKNEKNRPSLSLKTDNRPEKSKCKPLWGKVFYLDLPSVTI
Dbf4 motif-N
61 70 80 90 100 110 120
SEKLOKDIDKDLGGRVVEEFLSKDISYILSNKKEAKFAOTLGRISPVSPSPESAYTAETTSPH
121 130 140 150 160 170 180
PSHDGSSFKSPDTVCLSRGKLLVEKAIKDHDFIPSNLSNALSWGVKILHIDDIRYYIE
181 190 200 210 220 230 240
QKKKELYLLKKSSTSVRDGGKRVGSGAQKTRTGRLKKPFVKVEDMSQLYRPFYLQLTNMP
241 250 260 270 280 290 300
FINYSIQKPCSPFDVDKPSSMQKOTQVKLRIOTDGDKYGGTSIQLOLKEKKKKGYCECCL
Dbf4 motif-C
301 310 320 330 340 350 360
OKYEDLETHLLSEQHRNFAQSNQYQVVDDIVSKLVDFDVEYEKDTPKKKRIKYSVGSLSLP
361 370 380 390 400 410 420
VSASVLKKTEQKEKVELQHISQKDCQEDDTTVKEQNFYLYKETQETEKLLFISEPIPHPS
421 430 440 450 460 470 480
NELRGLNEKMSNKCSMLSTAEDDIRQNFTQLPLHKNKQECILDISEHTLSENDLEELRVD
481 490 500 510 520 530 540
HYKCNIQASVHVSDFDSTNSGSQPKQKSDTVLFPAKDLKEKDLHSIFTHDSGLITINSSQ
541 550 560 570 580 590 600
EHLTVQAKAPFHTPPEEPNECDFKNMDSLPSGKIHRKVKIILGRNRKENLEPNAEFDKRT
601 610 620 630 640 650 660
EFITQEENRICSSPVQSLDLDFQTSEEKSEFLGFTSYTEKSGICNVLDIWEEENSDNLTT
661 670 674
AFFSSPSTSTFTGF*

6/20

Fig.6



Dbf4 motif-N

H37 32: **LKTDNRF** **EKSK** **CKPL** **WGV** **FYLD** **PSVT** **IT** **CEK** **LQDK** **IK** **EL** **GGRV** **EFL** **SKD** **ISY** **L** **ISNKK** 91
 mu-H37 : **LKADNR** **LEKSK** **YKPL** **WGV** **FYLD** **PS** **IT** **CEK** **LQDK** **IK** **EL** **GGRV** **EFL** **SKD** **ISY** **FV** **SNKK**
 Dm-H37 : **TPPKV** **KV** **IKSK** **RPL** **CHFK** **FYLL** **ICDH** **QLAK** **RIES** **DIK** **AL** **GGH** **LE** **FFL** **SD** **IT** **FV** **TKP**
 *** ** ***** **

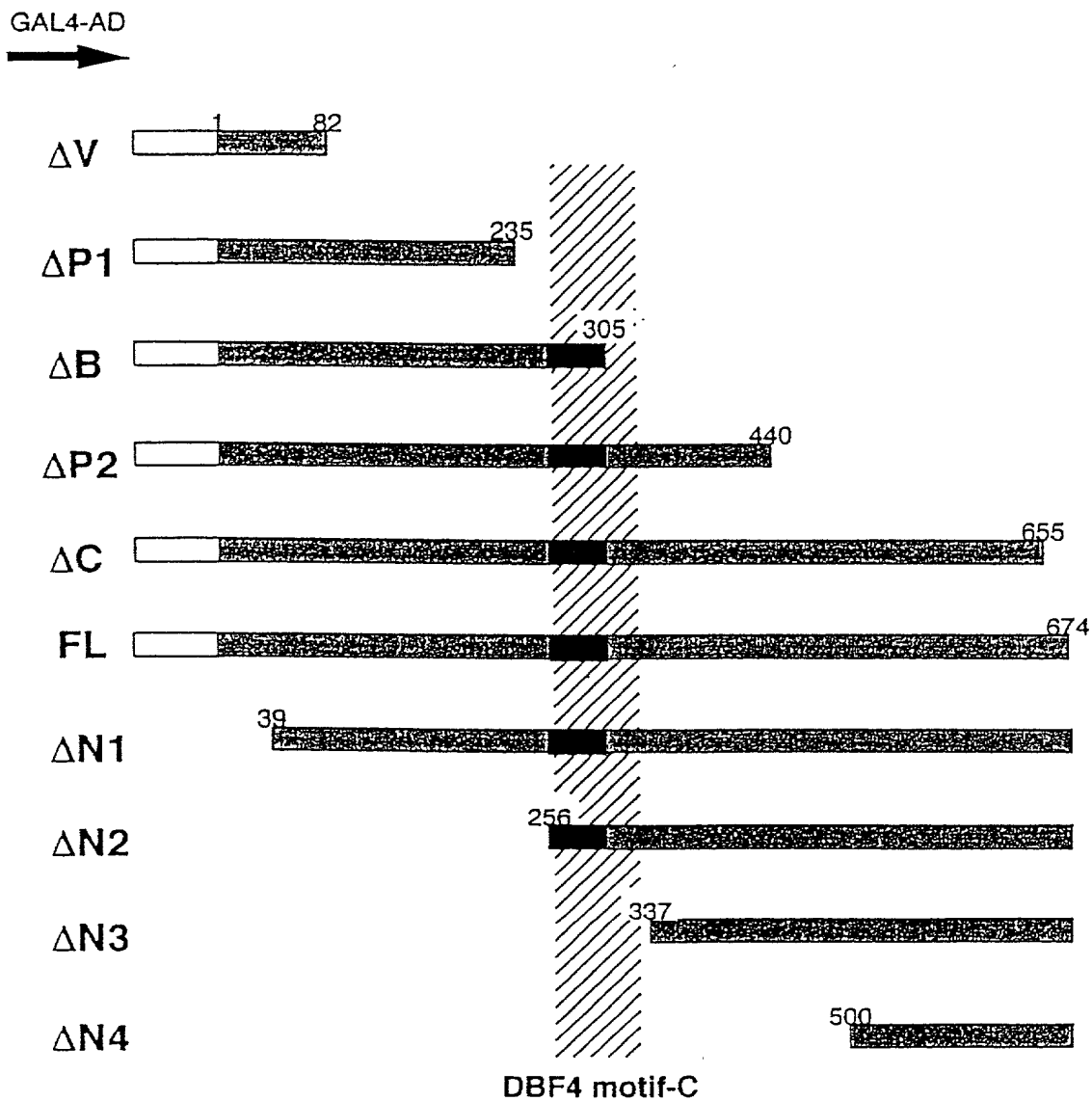
huDbf4N 92: **EAK** **FAQT** 98
 muDbf4N **EAK** **YAQT**
 DmDbf4N **EVIGGTS**
 *

Dbf4 motif-C

H37 263: **RQT** **QVKL** **RIQT** **DGKY** **GCT** **SIQL** **QLKE** **KKK** **GYCE** **CC** **LQKY** **ED** **LETH** **LSE** **CHRN** **FAQSN** 322
 mu-H37 : **KQAQ** **PKLR** **INMD** **GGKC** **GTPV** **QLQL** **KEKRR** **KGYCE** **CC** **LQKY** **ED** **LETH** **LSE** **CHRN** **FAQSN**
 Dm-H37 : **PSLQ** **ELKK** **QSAIP** **NSPR** **SNCRE** **PID** **SSE** **QCGV** **CE** **ICKL** **EYDI** **INIH** **LQSK** **DHEL** **FAKNS**
 Dbf4 619: **KKST** **STNV** **TLHF** **NAQ** **TACT** **AQPV** **KKET** **VKNS** **GYCH** **NRV** **KYES** **LE** **QHIV** **SE** **KHL** **SFAE** **N** 677
 * * * * *

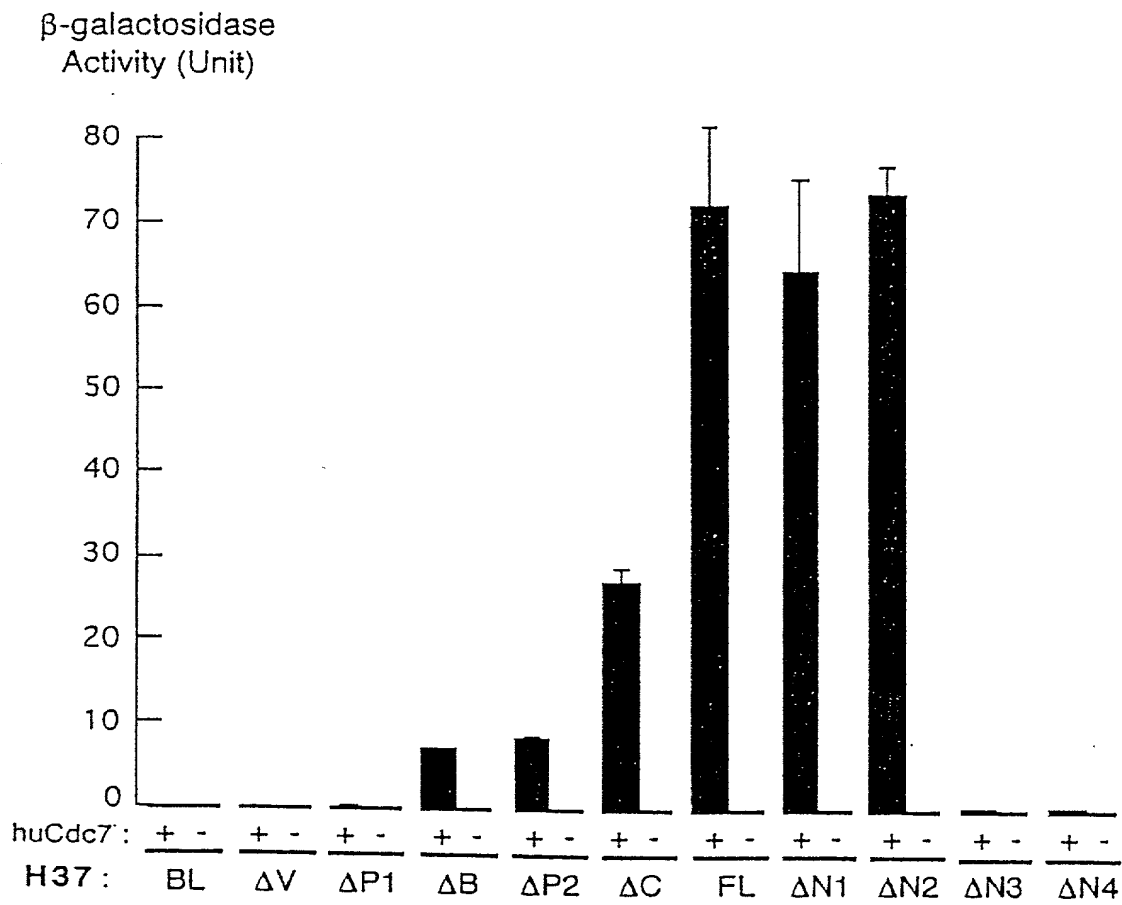
7/20

Fig.7



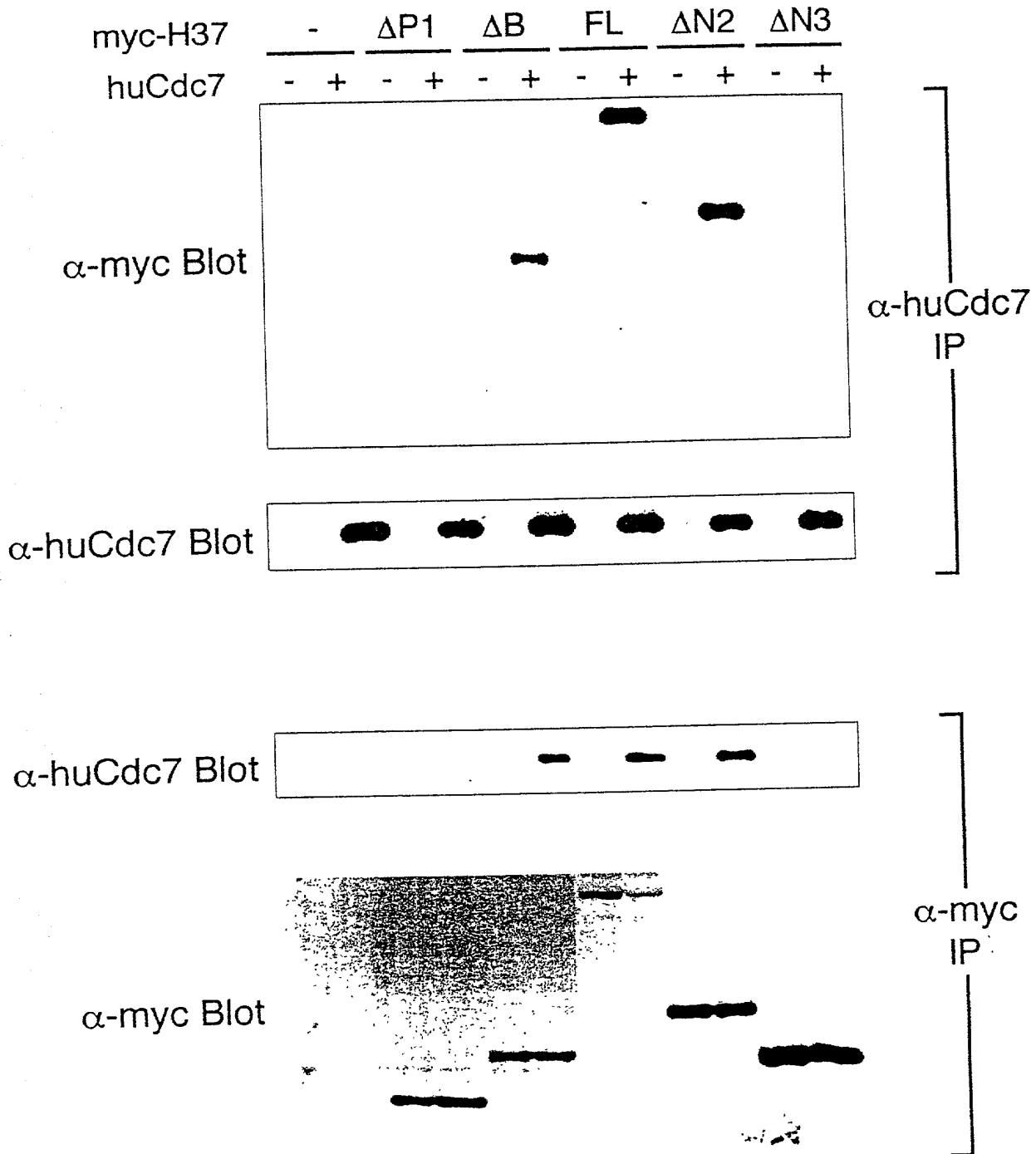
8/20

Fig.8



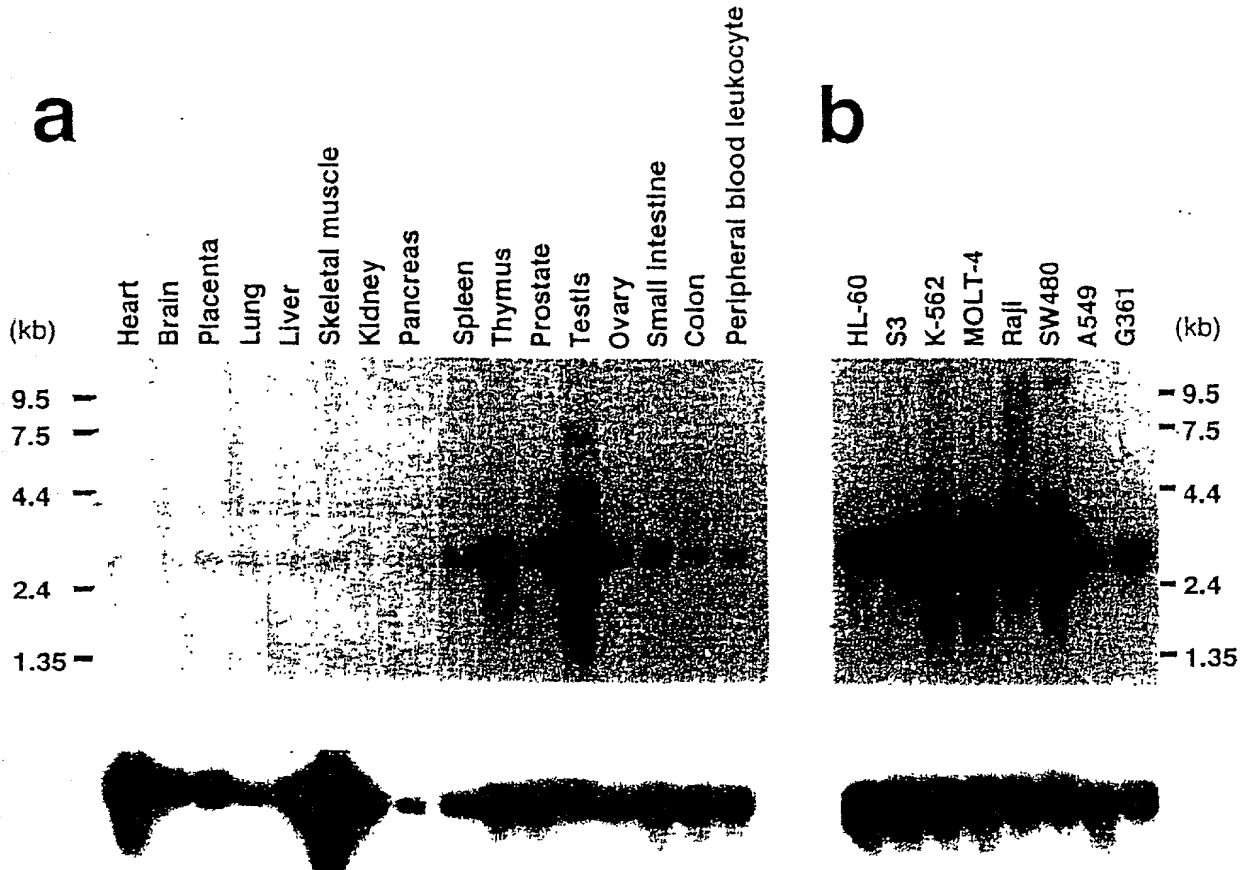
9/20

Fig.9



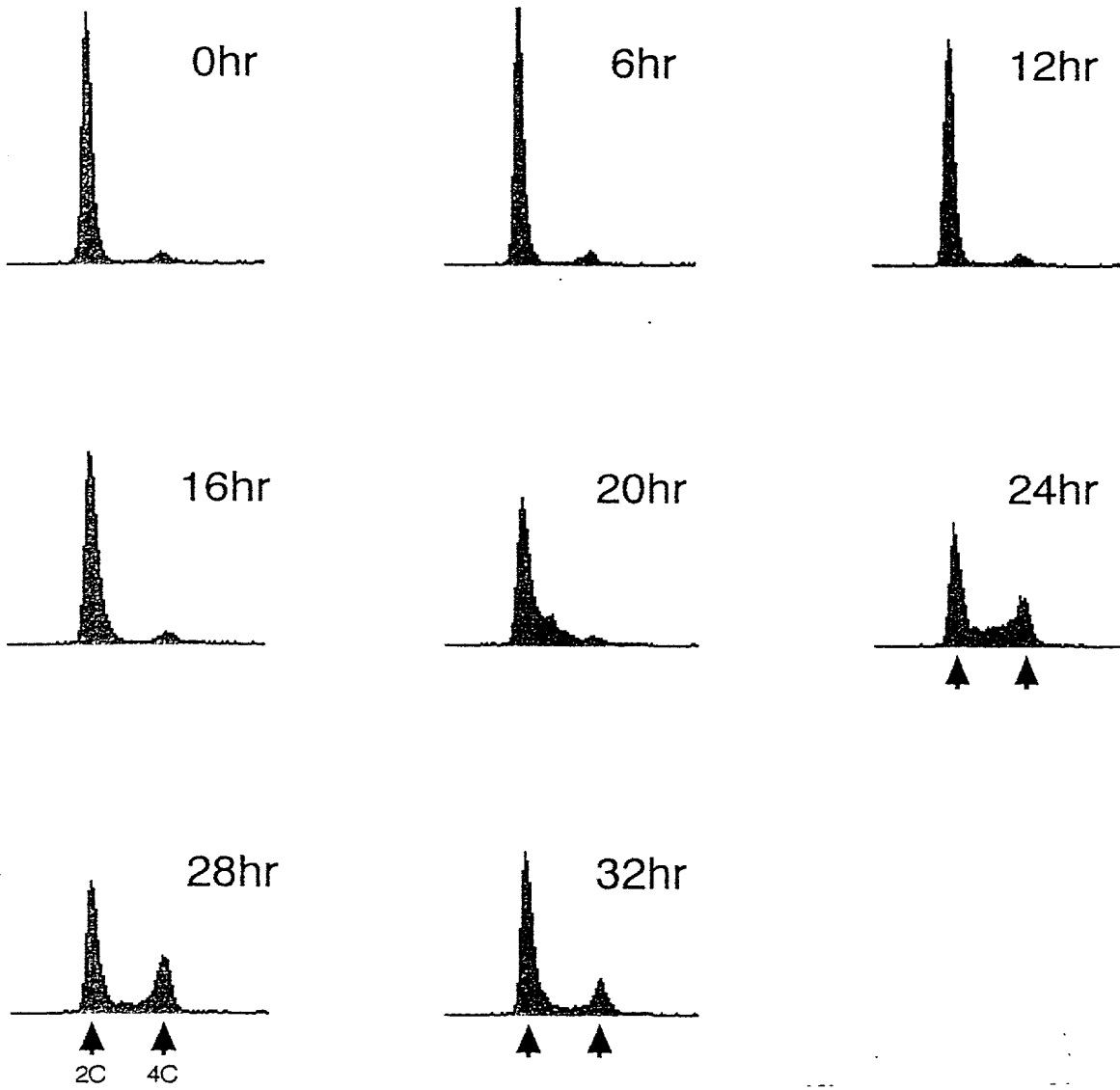
10/20

Fig.10



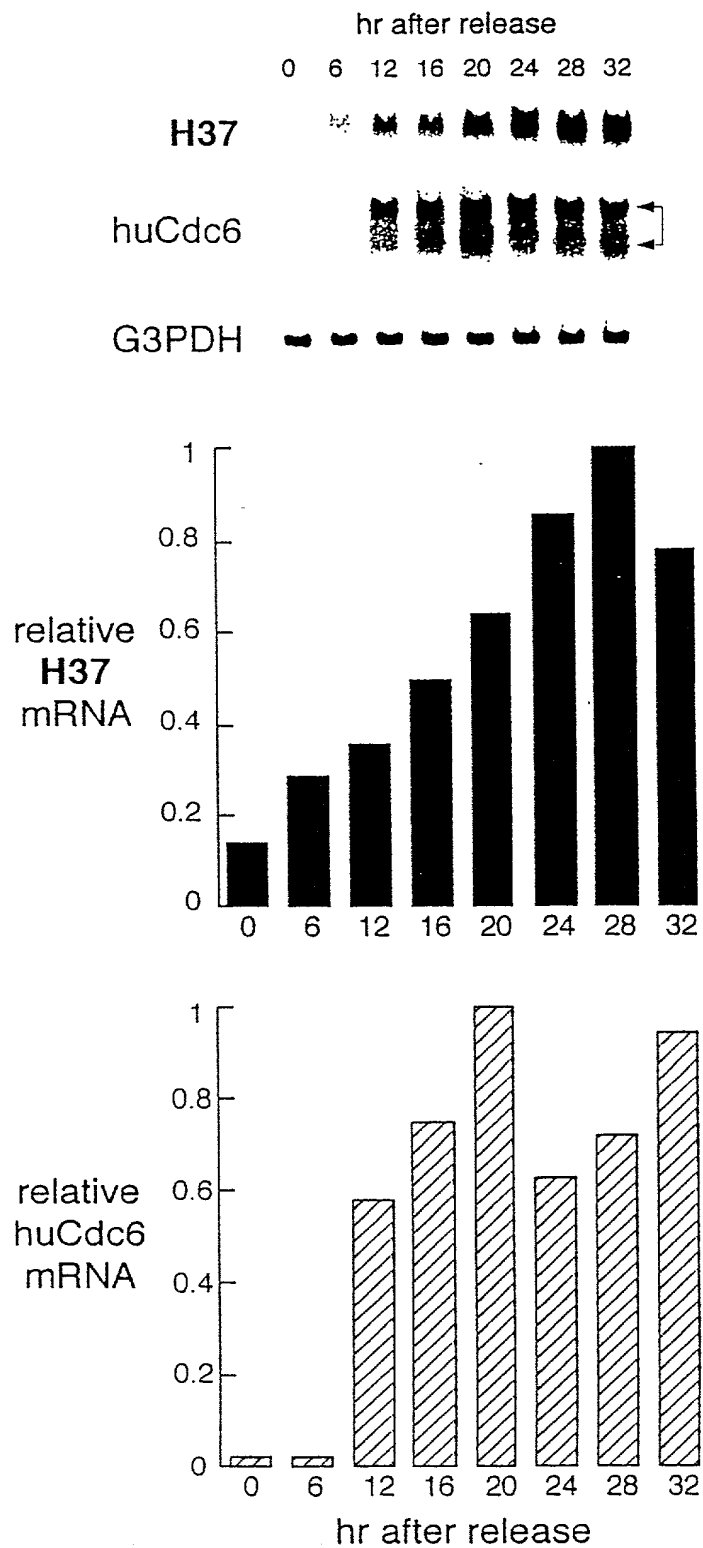
11/20

Fig.11



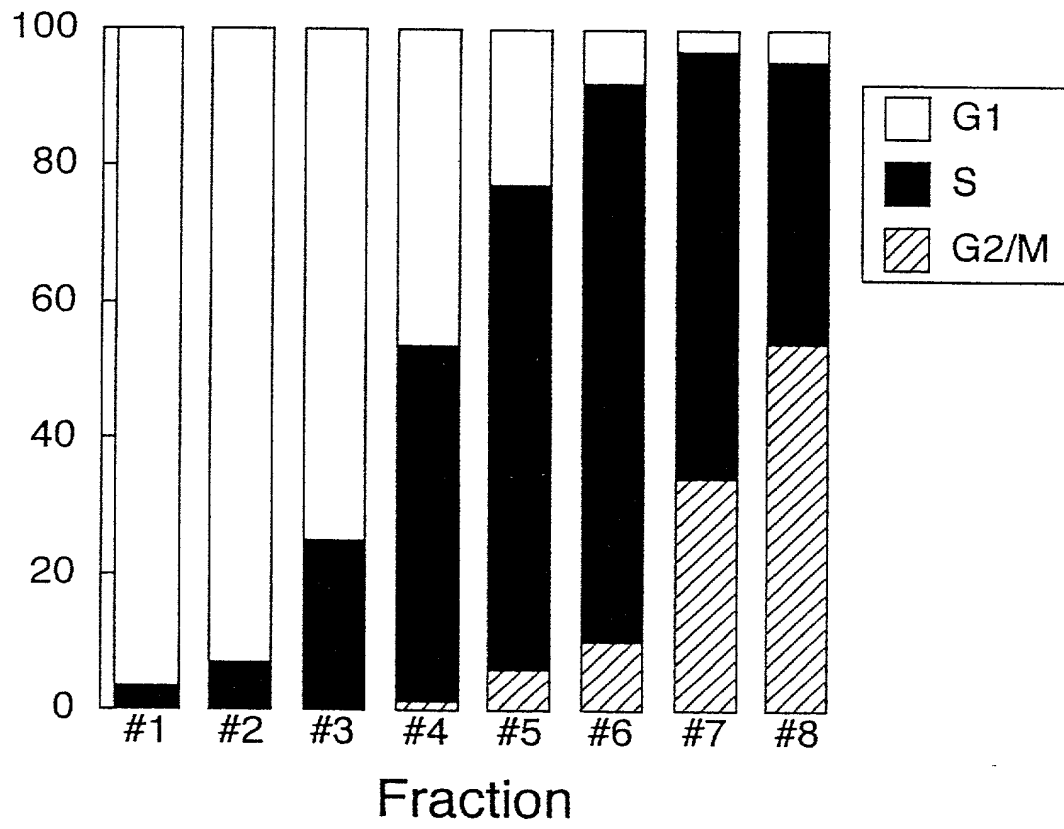
12/20

Fig.12



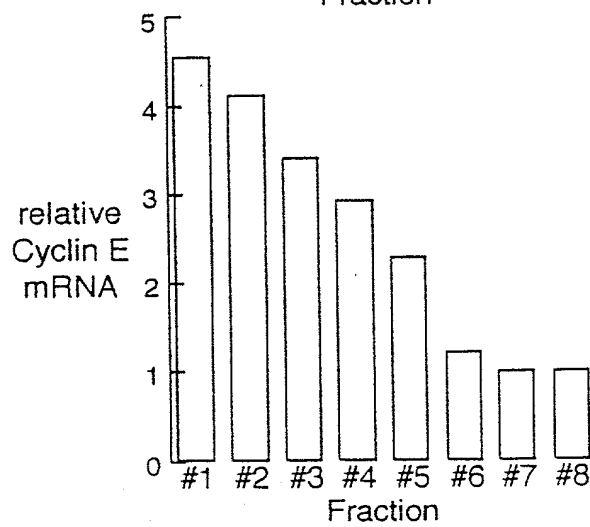
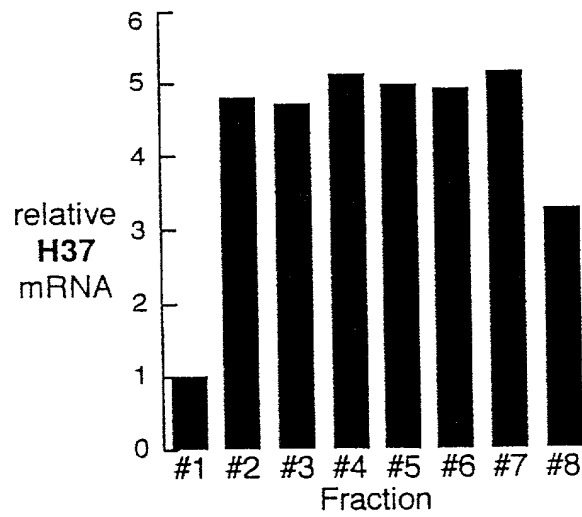
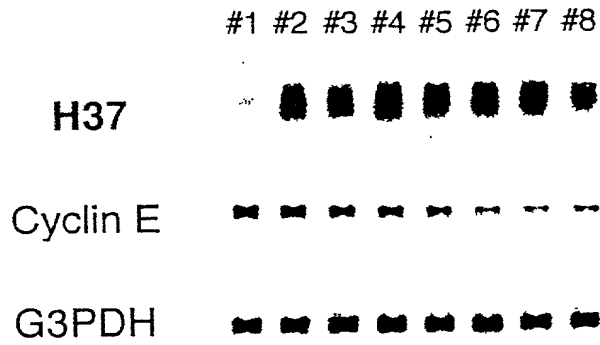
13/20

Fig.13



14/20

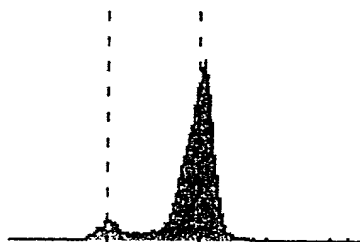
Fig.14



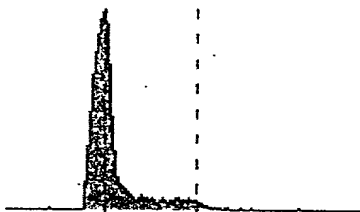
15/20

Fig.15

1 (G2/M)



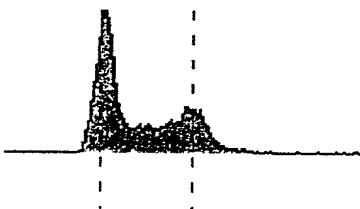
2 (6h)



3 (12h)



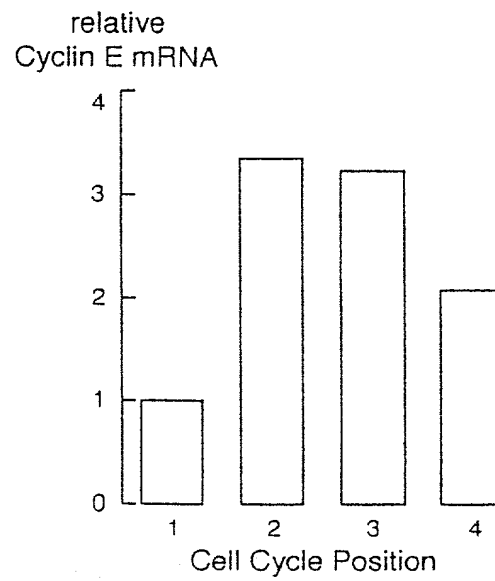
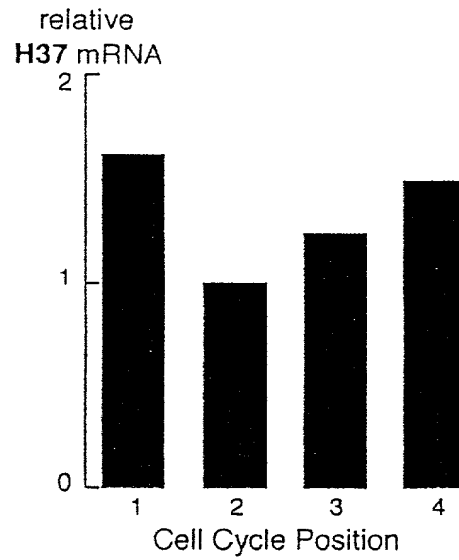
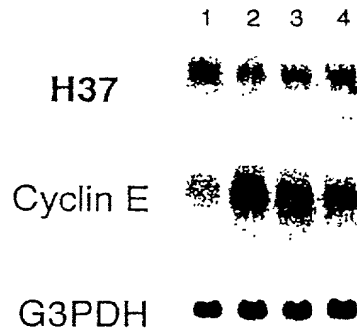
4 (18h)



09/830647 15/20

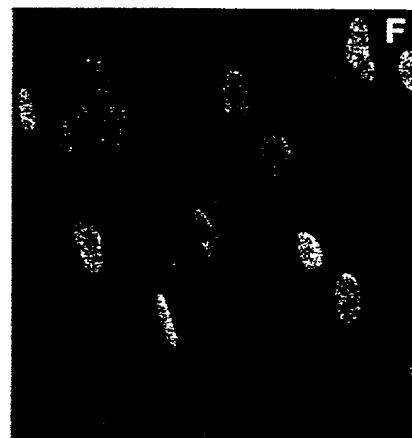
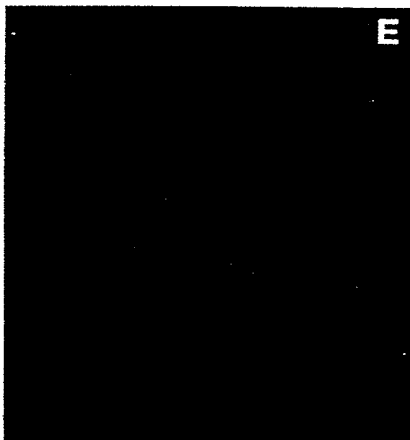
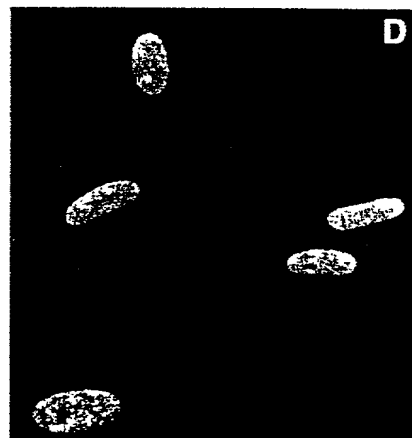
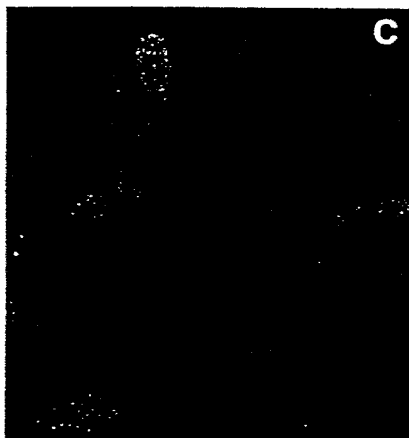
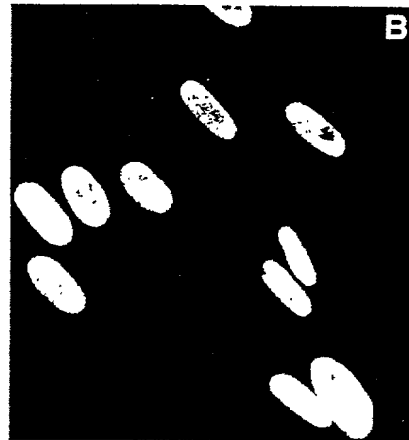
16/20

Fig.16



17/20

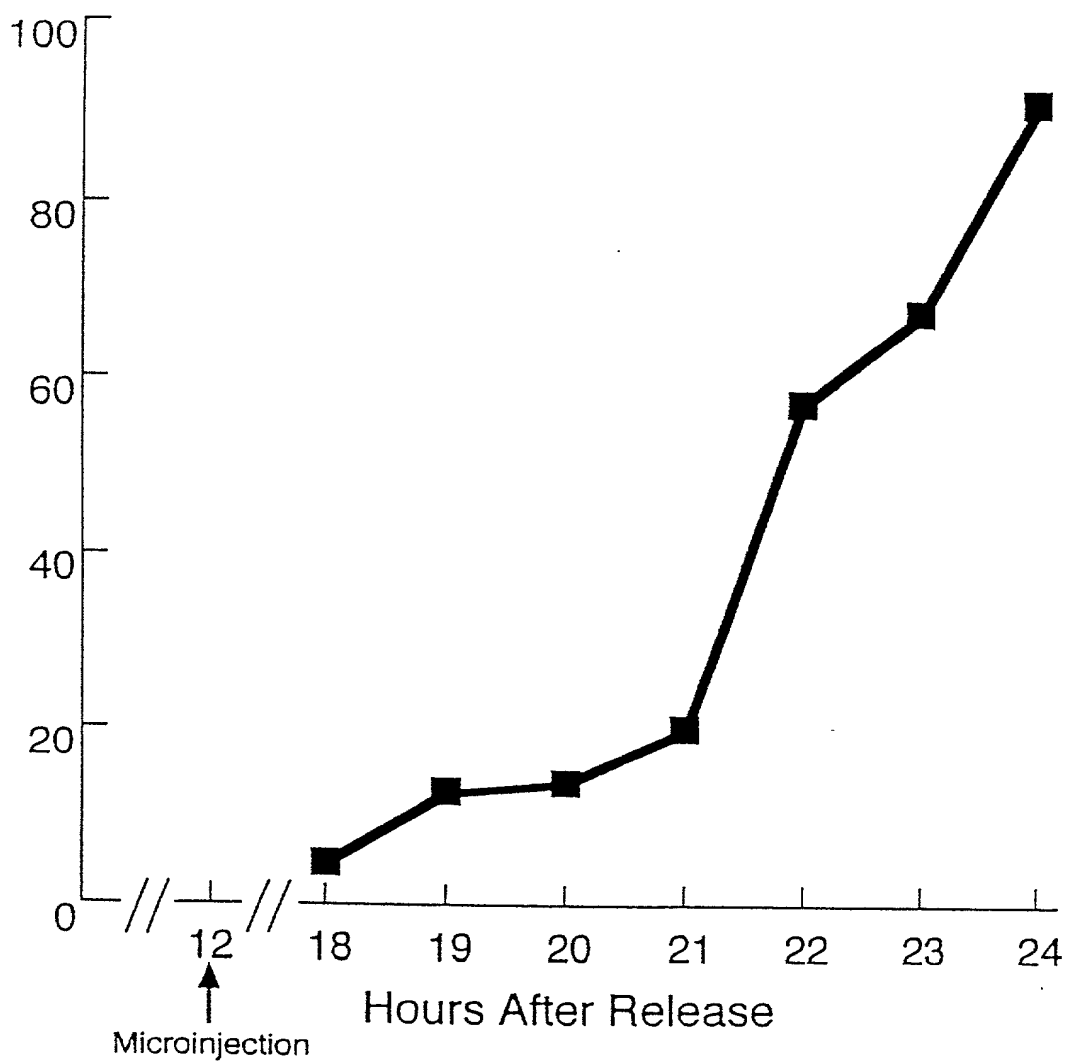
Fig.17



09/830647 17/20

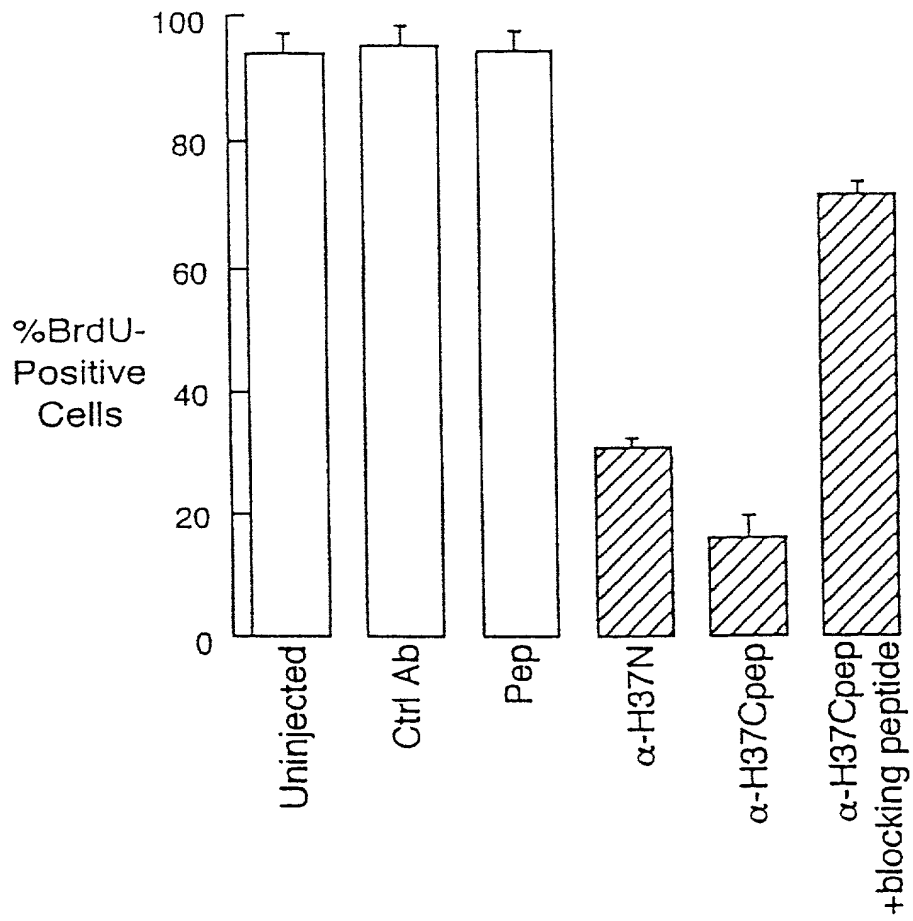
18/20

Fig.18

%BrdU-Positive
Cells

19/20

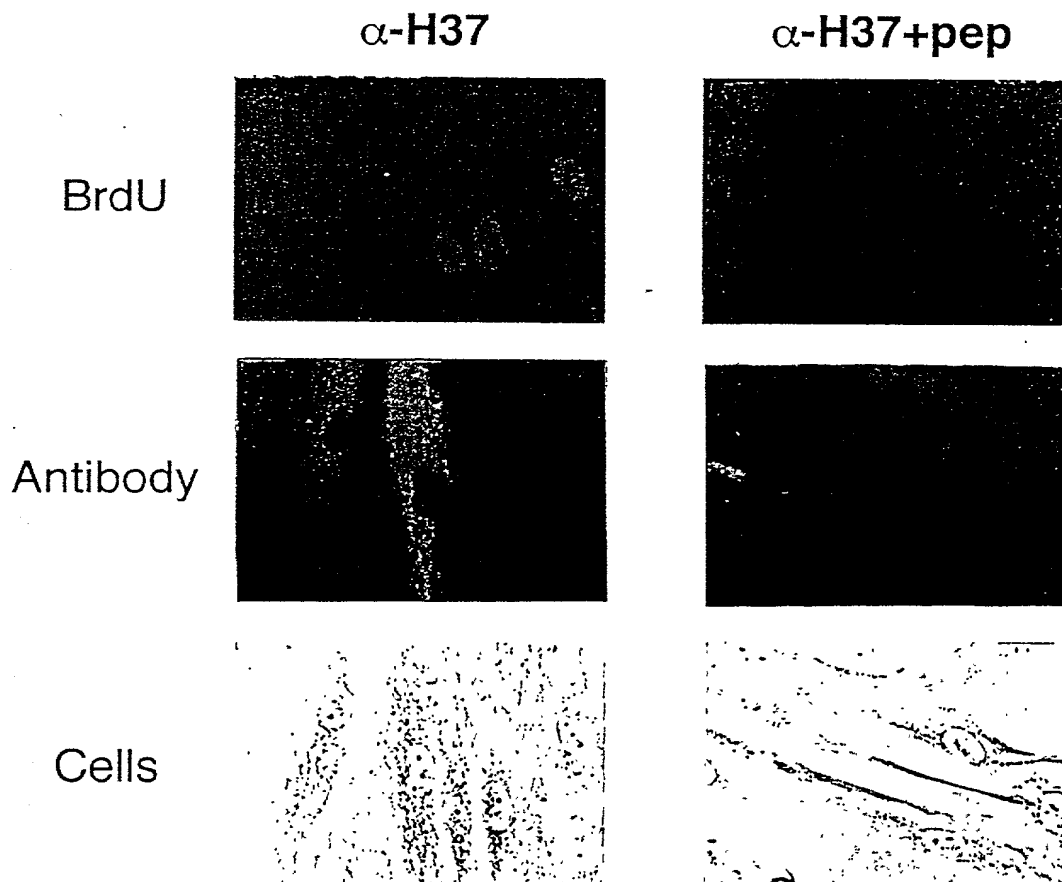
Fig. 19



20/20

Fig.20

Microinjection with



DECLARATION AND POWER OF ATTORNEY FOR U.S. PATENT APPLICATION

(X) Original () Supplemental () Substitute (X) PCT () DESIGN

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that I verily believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Title: HUMAN H37 PROTEIN AND cDNA ENCODING THE PROTEIN

of which is described and claimed in:

() the attached specification, or

(X) the specification in application Serial No. _____, filed April 30, 2001, and with amendments through _____, or

(X) the specification in International Application No. PCT/JP99/06076, filed November 1, 1999, and as amended on _____ (if applicable).

I hereby state that I have reviewed and understand the content of the above-identified specification, including the claims, as amended by any amendment(s) referred to above.

I acknowledge my duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim priority benefits under Title 35, United States Code, §119 (and §172 if this application is for a Design) of any application(s) for patent or inventor's certificate listed below and have also identified below any application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

COUNTRY	APPLICATION NO.	DATE OF FILING	PRIORITY CLAIMED
Japan	1998-311408	October 30, 1998	Yes

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	U.S. FILING DATE	STATUS: PATENTED, PENDING, ABANDONED

And I hereby appoint Michael R. Davis, Reg. No. 25,134; Matthew M. Jacob, Reg. No. 25,154; Warren M. Cheek, Jr., Reg. No. 33,367; Nils Pedersen, Reg. No. 33,145; Charles R. Watts, Reg. No. 33,142; and Michael S. Huppert, Reg. No. 40,268, who together constitute the firm of WENDEROTH, LIND & PONACK, L.L.P., as well as any other attorneys and agents associated with Customer No. 000513, to prosecute this application and to transact all business in the U.S. Patent and Trademark Office connected therewith.

I hereby authorize the U.S. attorneys and agents named herein to accept and follow instructions from NISHIZAWA & ASSOCIATES as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and myself. In the event of a change in the persons from whom instructions may be taken, the U.S. attorneys named herein will be so notified by me.

ATTACHMENT B

Direct Correspondence to Customer No:



000513

PATENT TRADEMARK OFFICE

Direct Telephone Calls to:

WENDEROTH, LIND & PONACK, LLP
2033 "K" Street, N.W., Suite 800
Washington, D.C. 20006-1021

Phone (202) 721-8200
Fax (202) 721-8250

Full Name of First Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	ARAI	Kenichi	
Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
	Tokyo	Japan JPX	Japan
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY ZIP CODE
	9-6-206, Meguro 1-chome, Meguro-ku, Tokyo, Japan		
Full Name of Second Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	MASAI	Hisao	
Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
	Tokyo	Japan JPX	Japan
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY ZIP CODE
	Shanbouru-Mita 620, Mita 5-7-8, Minato-ku, Tokyo, Japan		
Full Name of Third Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY ZIP CODE
Full Name of Fourth Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY ZIP CODE
Full Name of Fifth Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY ZIP CODE
Full Name of Sixth Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME

Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY ZIP CODE

I further declare that all statements made herein of my own knowledge are true, and that all statements on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1st Inventor Kenichi Arai Date May 25, 2001
 2nd Inventor Hisao MASAI Date May 28, 2001
 3rd Inventor _____ Date _____
 4th Inventor _____ Date _____
 5th Inventor _____ Date _____
 6th Inventor _____ Date _____

The above application may be more particularly identified as follows:

U.S. Application Serial No. _____ Filing Date April 30, 2001

Applicant Reference Number 99-F-031PCT-US/YS Atty Docket No. 2001_0531A

Title of Invention HUMAN H37 PROTEIN AND cDNA ENCODING THE PROTEIN